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AND BIOPHYSICAL CHEMISTRY

Volume 3

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EXTRACT FROM PREFACE TO VOLUME 1

THE editors have had some difficulty in deciding what is the proper field to be covered by reviews of recent progress in biophysics. Excluding biochemistry on the one hand and physiology on the other, there lies between a vast and rather amorphous field of study of which the frontiers and lines of demarcation are anything but well defined. On the one hand there is the application of the methods of physics to the study of the characteristic molecules of life—proteins, nucleoproteins, lipoproteins and other complex molecules of many and mostly unexplored varieties, a study which should perhaps be strictly regarded as an aspect of biophysical chemistry—hence the sub-title of the book. On the other hand there is the application of physical methods and instruments to the study and elucidation of living structures such as nerve and muscle fibres, a field in which modern refinements offer the greatest promise of progress in the future; progress which will perhaps bring its subjects into the first category. Both these fields of work are represented in the present volume and the editors have endeavoured to maintain a reasonable balance between them.

It has been our aim to obtain readable and interesting reviews of recent progress in selected subjects. Our contributors have been asked not merely to compile a précis of recent papers, or annotated lists of references, but to write critical reviews in which they discuss the subjects from their own point of view; which may be read with profit by many who are not experts and which will provide scientists with a general survey of recent work and ideas.

Since it is impossible in a single volume to deal at length with more than a few topics, it is intended that this volume will be followed by others at regular intervals. In time a more complete coverage of all the varied aspects of this rapidly developing branch of science will thus be built up.

May, 1950

J. A. V. BUTLER
J. T. RANDALL

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1

AUTORADIOGRAPHY

I. Doniach, Alma Howard and S. R. Pelc

AUTORADIOGRAPHY as a histochemical or cytochemical method is still somewhat novel, and its possibilities by no means fully explored. For this reason much of the emphasis in its application to biological and medical problems, even in very recent work, has been on the successful production of the autoradiograph, and the confirmation of previous findings, rather than on the harvesting of new knowledge from it. The number of fields in which autoradiography has been exploited as an experimental method is still quite limited. Outstanding among them are, first, the investigation of the distribution of heavy radioelements after administration. These studies have been of great importance in understanding the distribution of the damage due to the radiations emitted by these elements. Secondly, new knowledge on the growth and metabolism of bone has been gained. A third main field is the study of the thyroid gland by means of radioiodine. Much of the work up to 1950 has been covered in recent reviews and the present paper will emphasize recently published or unpublished work; the authors are grateful to those who have provided information on their work prior to publication or in the course of publication. GROSS and LEBLOND[1], GROSS *et al.*[2] and HELLER[3] have reviewed most of the reports in the literature concerning autoradiographs and the reader will find there summaries of the work on thyroid metabolism and bone formation, distribution of the actinide and lanthanide groups of elements, mustard gas labelled with radiosulphur and lewisite labelled with radioarsenic (AXELROD and HAMILTON[4]), radiosodium, potassium and copper, radioactive carbonate and glycine. HERZ[5] has reviewed the work on the physical and photographic principles of autoradiography.

PHYSICAL PRINCIPLES OF AUTORADIOGRAPHY

Autoradiography is a method for the detection and localization of radioactive material in a specimen by means of the photographic action of the ionising radiations emitted by the radioactive material. The processes involved in making an autoradiograph are : incorporation of radioactive material in a sample, preparation of the specimen, application of a photographic emulsion to the specimen, exposure, and photographic processing.

The aim of most experiments involving autoradiography is the study of the incorporation of radioactive material, and this part of the process therefore varies more than any other. The tracer is generally applied in a

chemical form different from that in which it is finally observed in the specimen. Any possible change in the chemical form has to be carefully considered in the design of experiments and in the interpretation of results. In biological experiments, the radiations emitted by the tracer whilst it is in the living organism will cause radiation damage which can influence the processes under observation and, in applications concerning humans, may produce undesirable effects. Evaluation of the radiation damage will be considered in the paragraphs on radiation dose and autoradiography. These also include a consideration of the possible changes in the distribution and amount of tracer during the preparation of the specimen.

The photographic action of ionising radiations is essentially the same as the photographic action of visible light; absorbed energy is utilized to transfer electrons from the crystal lattice of silver bromide crystals to that of silver sulphide specks. This results in the formation of small accumulations of silver, by the reduction of silver sulphide to silver, which act as a catalyst for the developer. The silver bromide crystals carrying the catalyst are reduced to metallic silver whilst those which are in the original state are not affected. During fixation the unreduced silver bromide is dissolved, and the remaining developed grains form an image of the pattern of the radiation impinging on the emulsion. It should be noted that photographic emulsions are not genuine emulsions but consist of silver bromide crystals embedded in gelatine. A certain number of grains is always developed due to chemical imperfections, cosmic radiation and exposure to light during manufacture and handling of the emulsion in the darkroom. This background has to be kept as low as possible and should be much lower than in ordinary photographic practice. Photographic emulsions are much less sensitive to γ-rays than to β-rays and therefore most of the theoretical work has been based on the assumption that β-emitters are used. The preparation of α-track autoradiographs has been described by LEVI[118].

The most important points in autoradiography are : the speed of the process *i.e.* the number and size of grains developed per ionising particle hitting the film, the resolving power *i.e.* the detail which can be observed, and the background and absence of artefacts which can be mistaken for grains. The optical density of the autoradiograph is the criterion when little detail is required, whilst concentrations of single grains are observed in preparations of high resolving power. These two kinds of autoradiograph need different theoretical treatments.

HERZ[5] has reviewed the available experimental data for speed of emulsions. To obtain a density of 0·6 with 15 days exposure on a fast non-screen X-ray film, 3·6 to 21 $\times$ 10^{-4} $\mu C/cm^2$ of radioactive material are necessary. This range is valid for C^{14} emitting β-particles with a maximum energy of 0·154 MeV to P^{32} with 1·7 MeV. The number of β-particles

hitting one sq. cm of film during this exposure varies from 0·38 to $7{\cdot}2 \times 10^7$. For autoradiographs of low resolving power a density of 0·6 is usually higher than necessary, and since the tracer will usually be broken up into a number of foci, the total activity, as shown by measurements of a section with the G-M counter, could be well below the quoted figures. Extensive measurements of the response of photographic emulsions to β-particles were carried out by DUDLEY[6].

When high resolving power is required, the number of grains per electron rather than the optical density can be regarded as the significant figure. HERZ[5] and LAMERTON[7] found for stripping film for autoradiography (*Kodak Ltd.*) grain yields of 0·5 to 0·77 per β-particle for P^{32} and 1·8 for S^{35}. The background on this film can be as low as 4 grains per 100 μ^2. HOWARD and PELC[8] counted the minimum concentration of grains above background which can be observed as an autoradiograph as 10 grains per 100 μ^2. 100 μ^2 is approximately the area covered by a small cell or a large nucleus and has been chosen as the unit area for this reason. The minimum of 10 grains per 100 μ^2 would not be valid for much smaller areas since, for example, 1 grain per chromosome covering 10 μ^2 would be indistinguishable from background. Statistical methods could of course be used to increase the sensitivity. This figure shows however the great sensitivity of the method. Assuming a grain yield of one, 10 grains above background are due to the decay of 20 atoms during the exposure, since one half of the β-particles will be emitted in a direction opposite to the emulsion. For exposures of two half-lives when 75 per cent of the tracer present will have decayed, the method permits the detection of 27 atoms of radioactive material in a cell nucleus.

MARINELLI and HILL[9] and ODEBLAD[10] suggested measuring the total activity in a specimen with a G-M counter from which the correct exposure time can be determined from equations taking into account sensitivity of emulsion, decay, and distribution of activity. The method is of course useful only where the approximate distribution of radioactive material is known. In the present authors' experience the best method is by trial and error *i.e.* developing one preparation after short exposure, from which the correct exposure time can be estimated.

Absolute estimates of the amount of tracer incorporated by comparison of the optical density of autoradiograph and a test exposure with a source of known content were reported by AXELROD and HAMILTON[4] and DUDLEY and DOBYNS[11]. This method is, on account of the relatively high densities employed, unsuited for high resolution work where the underlying specimen would be practically obliterated by an autoradiograph of a density of say 0·2. For this reason, and to preserve resolving power, much lower densities must be used and grain counting becomes necessary. Automatic grain counters are under construction and will be of great value

for further progress. The foundations for quantitative autoradiography are now sufficient for extensive work along this line.

Resolving power could satisfactorily be defined as the shortest distance between two point sources of radiation which result in two distinct images. This definition does not lend itself to mathematical treatment since the term "two distinct images" would have to be defined. DONIACH and PELC[12] used the half width of the curve describing the response of the photographic emulsion as a measure of the resolving power. It was found that values calculated using this definition agree well with experimental results. The scheme adopted is shown in Figure 1. If Q is a point source of radiation at a depth x in the specimen of thickness d and P any point

Figure 1. Schematic representation of source and emulsion in autoradiography.

in the emulsion of thickness a separated from the specimen by a distance δ, the intensity of radiation at P will depend on the distance r between P and Q. β-particles will be emitted at Q in all directions and their progress will be affected by scatter and absorption. It would be almost impossible to express these processes in a comprehensive mathematical form and, for the purpose of these calculations, it is assumed that they proceed in straight lines. For low densities the number of grains made developable is proportional to the number of β-particles crossing an element of space in the emulsion. If M is the number of β-particles per unit time emitted at Q the number of developed grains at P will be:

$$dn = \frac{M}{4\pi r^2}\, dr \qquad \ldots . (1)$$

The total number of grains in the section through the emulsion at P will be

$$n = \frac{M}{4\pi} \int_{y=0}^{a} \frac{dr}{r^2} \qquad \ldots . (2)$$

A point source might be at any depth in the specimen, the adoption of a line source subtended through the specimen instead of the point source gives an average for point sources at various depths. The number of grains in a section of the emulsion at the distance z from the perpendicular is:

$$n = \frac{M}{4\pi} \int_{y=0}^{a} dy \int_{x=0}^{d} \frac{1}{z^2 + (y + \delta + x)^2}\, dx \qquad \ldots . (3)$$

The resolving power depends also on the grain size and cannot be better than approximately twice the grain diameter. This value has to be added to the calculated figure.

Computations of equation (3) are shown in text Figure 2 and *Table I.*

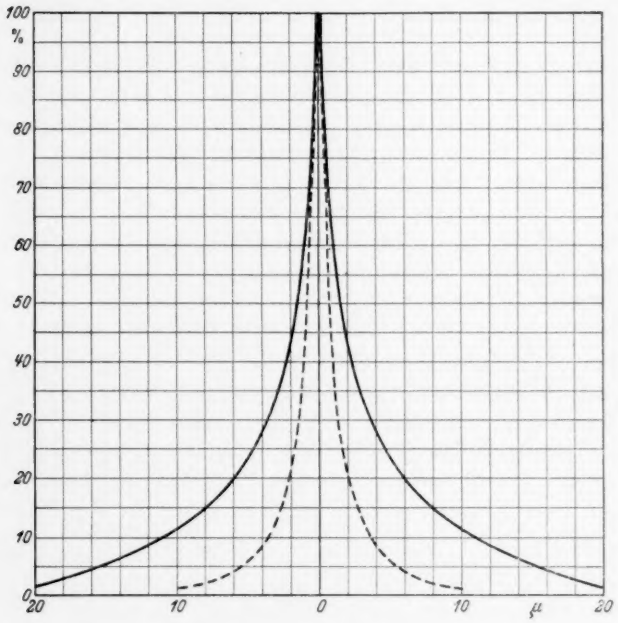


Figure 2. Resolving power computed from equation (3).
Abscissae : Z (Figure 1) in microns.
Ordinate : response of emulsion
X ....d =5 μ, a = 15 μ, δ = 0.1 μ.
O ....d = 2 μ, a = 2 μ, δ = 0.1 μ.

The narrower curve in Figure 2 was computed assuming a thickness of 2 μ for emulsion and specimen to give a half width of 2 μ. The wider curve is for an emulsion 15 μ thick and a specimen of 5 μ with a half width of 3·5 μ. The difference in half width might be of little importance for some applications but the much slower flattening of the wider curve shows that much crisper images can be expected when thin specimen and emulsions are used. *Table I* demonstrates the influence of separation on the resolving power. On the same assumptions NADLER[13] calculated the resolving power for evenly loaded disc sources, as an approximation to the shape of the colloid masses in thyroid sections.

Table I. Resolving power, all figures in microns

Thickness of		*Resolving power*		
Specimen d	*Emulsion* a	$\delta = \cdot 1$	$\delta = 1$	$\delta = 3$
5	15	3	9	17
2	2	2	5	—
2	5	2	5·5	10

Experimental work confirmed the conclusions drawn from these calculations. STEVENS[14,15] converted microimages of geometrical patterns on photographic plates to silver iodide using I^{131} and used these as specimens for the production of autoradiographs under varying conditions. GROSS *et al.*[2] and NADLER[13] counted the number of grains in cross-sections of preparations. Visual results on biological specimens by ODEBLAD[10] and the present authors' results also confirm this theory. The β-ray energy becomes of importance for maximum energies below 0·2 MeV.

RADIATION DOSE AND AUTORADIOGRAPHY

The ionising radiations emitted by the tracer whilst it is in a living organism are capable of causing damage and may influence the processes under investigation. The wide variation in the sensitivity of tissues to radiations necessitates special consideration of this aspect for each experiment. The radiation dose in experiments involving autoradiography can be calculated[16] if sufficient data for uptake and excretion of the tracer are available. Some tracer will usually be lost during preparation of the specimen or extracted to retain only the fraction incorporated in certain compounds; the radiation dose will however be caused by the total amount of tracer present.

The concentration of a radioactive element (a_τ) in μC/cc at the time τ, (δ) if A μC/cc was given at time O, will be:

$$a_\tau = A f(\tau) e^{-\lambda\tau} \quad \mu C/\text{cc} \qquad \ldots . (4)$$

where $f(\tau)$ describes the change in concentration with time for a stable isotope; the last term in equation (4) takes radioactive decay into account. Assuming that the sample for autoradiography is removed at time t and a concentration C is needed to obtain an autoradiograph after a suitable time of exposure, the amount of tracer to be applied at time O will have to be:

$$A_0 = \frac{C}{f(t)} e^{\lambda t} \quad \mu C/\text{cc} \qquad \ldots . (5)$$

The concentration C can be calculated from the known sensitivity to β-particles of the photographic emulsion, from the number of developed

grains necessary for observation and from the ratio of radioactive volume to total volume (f).

For an element with a half-life H in days and an exposure time of two half-lives, the minimum concentrations at the time the specimen is removed are, for special stripping film and x-ray film:

$$C_s = \frac{23f}{H} \quad \text{and} \quad C_x = \frac{0{\cdot}6f}{H} \qquad \ldots . \text{(6a, b)}$$

If the dimensions of an organ are large compared with the range of electrons emitted, the dose rate will be:

$$d.r. = 2{\cdot}54\, a\, \bar{E}_\beta \qquad \text{reps per hour} \qquad \ldots . (7)$$

where a is the concentration in μC per cc and $\bar{E}_\beta$ the average energy of the electrons in MeV. LAMERTON and HARRISS[17] calculated the difference in dosage for evenly distributed and highly concentrated material, especially when the original radioactive material was in particulate form.

Combining equations (5), (6), (7) and (8) we obtain for the dose-rate using stripping film:

$$d.r. = 58{\cdot}4\, \frac{f\bar{E}_\beta}{H\, f(t)}\, e^{\lambda t}\, f(\tau)\, e^{-\lambda\tau}\, d\tau \text{ reps per hour} \qquad \ldots . (8)$$

The total dose to the organ up to the time T is the integral of the dose-rate

$$d = \frac{58{\cdot}4\, f\, e^{\lambda t}\, \bar{E}_\beta}{H\, f(t)} \int_{\tau=0}^{T} f(\tau)\, e^{-\lambda\tau}\, d\tau \quad \text{reps} \qquad \ldots . (9)$$

In many cases the dose given to an organ different from the one under investigation may be of importance, in which case $f(\tau)$ will generally be a different function. Equation (9) can be solved for a variety of functions $f(\tau)$. When using these equations tracer lost during the preparation of the specimen must be taken into account. In general, the concentration of the isotope in an organ will increase and subsequently some will be lost by excretion. An approximate fit for this rise and fall of the concentration is sometimes obtained using $f(\tau) = b\tau e^{\alpha\tau}$ for which equation (10) can be integrated.

Techniques of autoradiography

Many of the technical problems of autoradiography are unsolved and the technique to be used for a certain experiment depends on, and has to be adapted to, the particular needs.

For autoradiographs of low resolving power the original technique of pressing a piece of film against the specimen is widely used[18]. Using fast

x-ray film an exposure of 10^6 — 10^7 β-particles per sq. cm is sufficient, the resolving power will be of the order of 50 μ, Figure 3. The resolving power is better if flexible stripping film is used. This high speed will be utilized only when a reasonably even distribution of tracer prevails in the specimen; for sparsely distributed cells one of the methods giving higher resolving power and less background can be more sensitive. The carbowax technique[19,20] and freeze drying techniques[21,22,23] have proved useful when water soluble compounds are to be retained.

BÉLANGER and LEBLOND[27] obtained similar resolving power by melting photographic emulsion at 37°C and painting it on a specimen covered with a thin layer of celloidin, Figure 4. The method has been improved in BÉLANGER'S[28] inversion technique, Figure 5. The specimen plus photographic emulsion are inverted after photographic processing and can then be stained, thus obviating the danger of shifting radioactive material during staining before autoradiography. LEVI[115] succeeded in re-inverting the preparation after staining to improve observation of the autoradiographs.

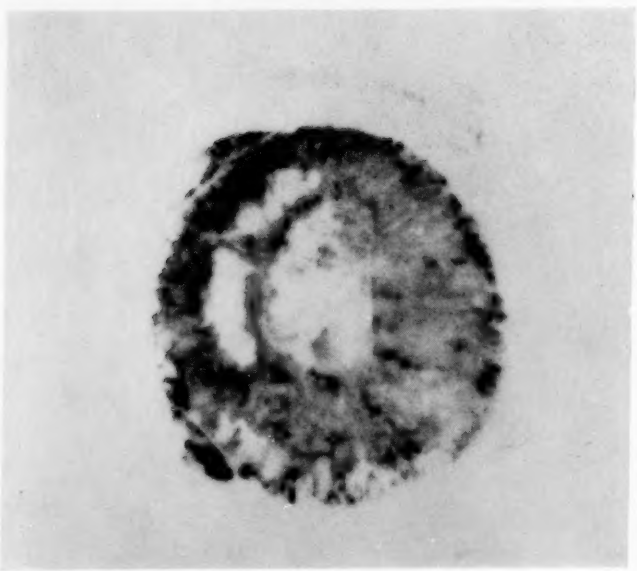
The mounting method was developed independently by ENDICOTT and YAGODA[24] and EVANS[25]. Sections are floated on to a photographic plate, dried and stored for exposure. After exposure the paraffin wax is dissolved in xylene and the preparation developed and fixed; it can then be stained. The resolving power is about 5-7μ. BOYD and WILLIAMS[26] used stripping film backed by cellulose acetate instead of plates (the cellulose acetate facing the specimen) and succeeded in avoiding artefacts due to substances diffusing into the emulsion from the specimen.

Stripping emulsion backed by pure gelatine floated on distilled water and coated on to a thin specimen[29,12] gives a resolving power of 2-3 μ with P^{32} and 1-1·5 μ with soft emitters like S^{35} when special stripping film is used[30], Figure 6. The specimen can be stained with Feulgen or hot fuchsin before processing, or with haematoxylin (Figure 7) or Giemsa afterwards, Figure 8. Observation of unstained specimens by phase contrast is the method of choice because it allows an independent view of specimen and autoradiograph.

Recently attempts have been made to use nuclear track emulsions sufficiently sensitive to give electron tracks[31,32,33], Figure 9. The best resolving power so far claimed for this method is 5 μ; the sensitivity is higher than that of other methods for short exposure times or very short lived isotopes. For long exposures the background due to cosmic rays is a severe limitation. LEVI[115] found that some of these nuclear track emulsions are inhomogeneous.

Attempts to adapt the wet collodion process for autoradiography by GOMBERG[34] are still in the experimental stage. In this process a very thin film of extremely fine grain is formed on the specimen and then developed

(*a*)



(*b*)

Figure 3. Roentgenogram (*a*) and gross autoradiograph (*b*) of thick section of an osteogenic sarcoma. Female, age 12. Ga^{72}, 2 days before disarticulation of left leg. (Copyright, *Oak Ridge Institute of Nuclear Studies, Oak Ridge, Tennessee, U.S.A.*) × 1/2

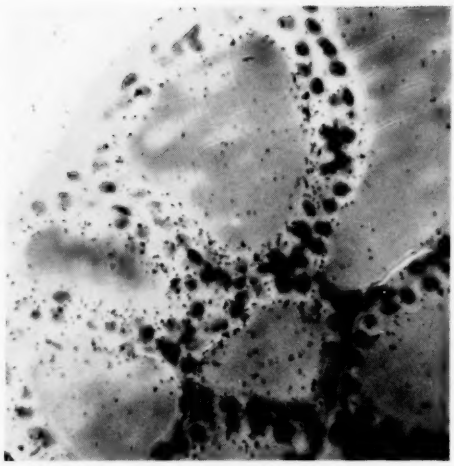
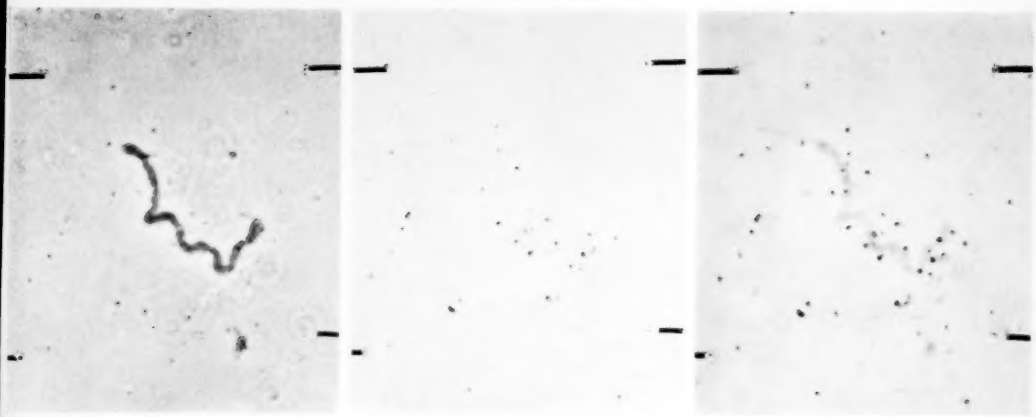


Figure 4. Coated autoradiograph of a section of thyroid from a rat kept on a high iodine diet. 20μC I^{131} for one hour. Haematoxylin and eosin stain. (From GROSS *et al.*[2])

Figure 5. Portion of a cusp of the 1st molar, upper jaw, in a Hamster of 6 days of age, injected 2 days previously with a single tracer dose of P^{32}. The autoradiographic picture indicates the presence of the radioelement in the dentine and the enamel. In the dentine, a radioactive line exists at a small distance from the odontoblasts. There is a larger amount of P^{32} at the apex where growth is maximal in this tissue. In the enamel, the autoradiographic picture indicates a distal pattern of deposition. In the more mineralized area of the apex the P^{32} seems to be present in a portion of the enamel which is at some distance from the enamel-dentine junction. In the less mineralized lateral regions of enamel, the 'reaction' approximates the dentino-enamel junction.

× 100, celloidin section of 10 micra; basic fuchsin stain following inversion. Ref. BELANGER, LEONARD, F., Autoradiographic and histochemical observations of the phenomenon of mineralization in Teeth of Rats and Hamsters of various ages (unpublished).



(*a*) (*b*) (*c*)

Figure 6. Chromosome from *Vicia faba* (broadbean) root squash.
Root grown for 20 hours in S^{35}.
(*a*) phase contrast, (*b*) no phase contrast (same field), (*c*) *a* and *b* superimposed.
× 1300

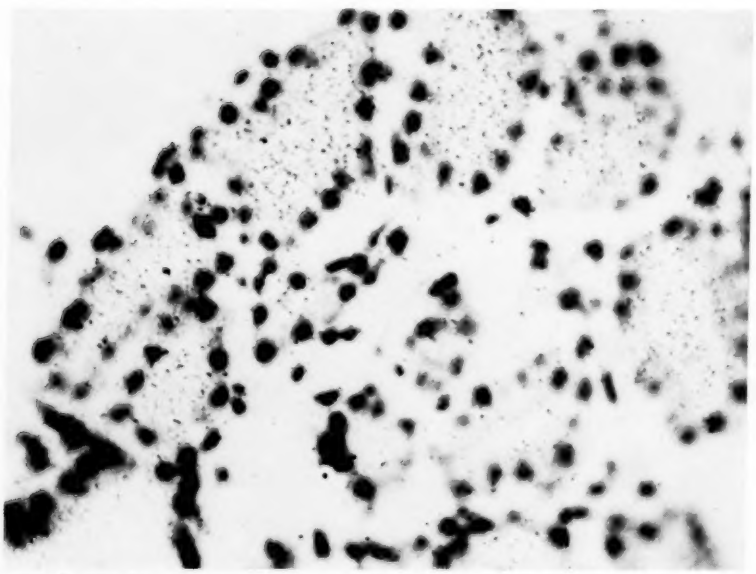


Figure 7. Stripping film autoradiograph of a rat thyroid, I^{131}, stained with haematoxylin. Stripping film technique. Focussed on grains. × 400

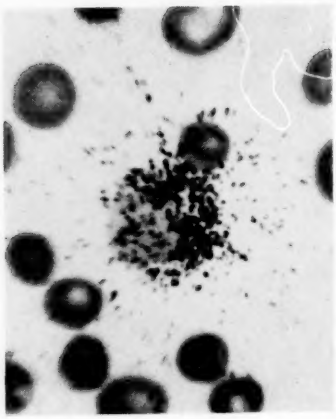


Figure 8. Smear of human bone marrow grown in vitro in 1 μC/cc S^{35} for 24 hours. Stripping film technique. Stained with watery Giemsa. The myelocyte shows significantly higher concentration of S^{35} in the cytoplasm than in the nucleus. (Courtesy of Dr. L. G. LAJTHA).

Figure 9. A specimen of *Paramecium caudatum* cultured in P^{32}. Tracks are seen radiating outward from a food vacuole at the anterior end.
(KING, D. T., TKACZYK, S. and HARRIS, J. E. *Proc. Radioisotope Techniques Conference*, Oxford, July 1951.)

Figure 10(*a*). Stripping film autoradiograph of a thyroid section, I^{131}. Thyrotoxicosis. Showing different uptake in follicles. Phase contrast.

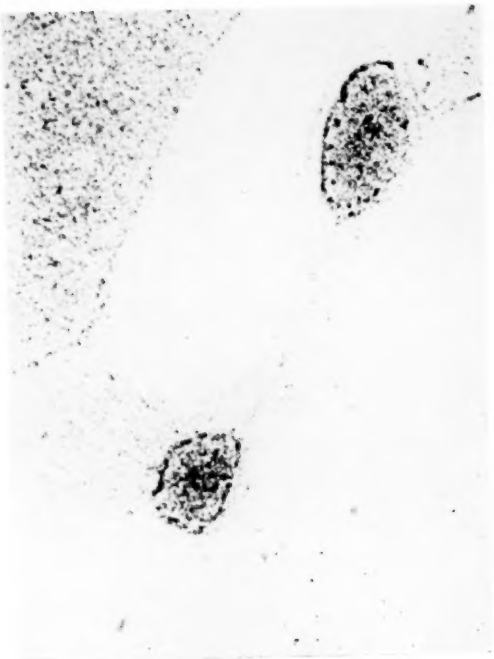
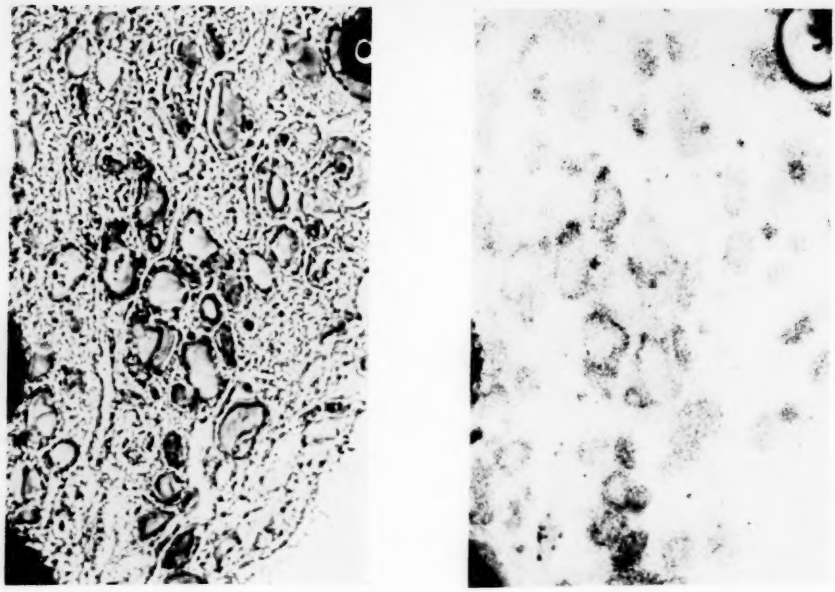


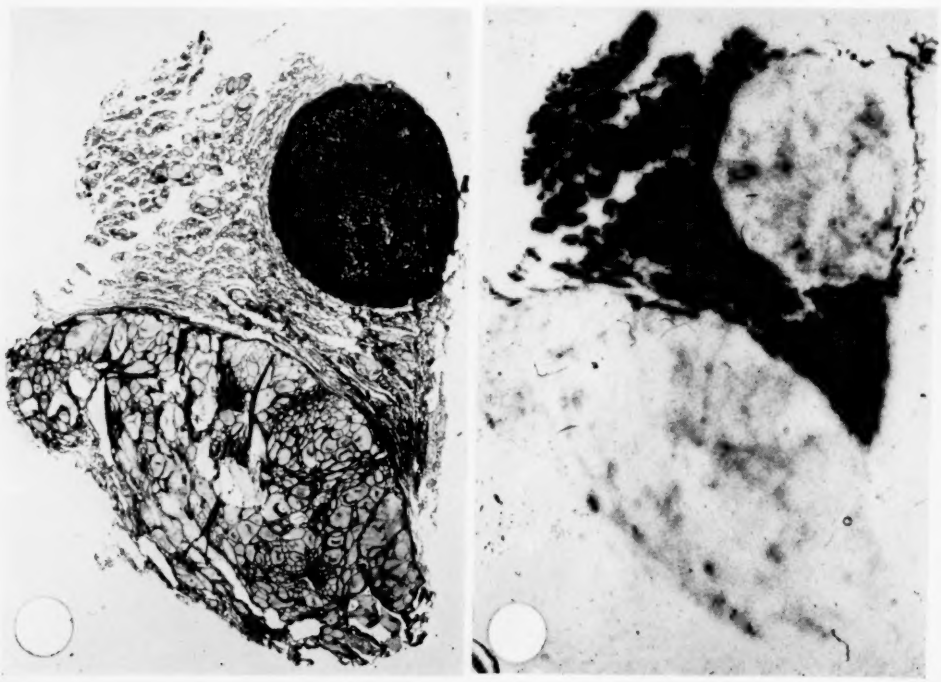
Figure 10(*b*). Same field as 10(*a*), no phase contrast.

× 200



(*a*) (*b*)

Figure 11. Section of a rat thyroid after 6 weeks' treatment with thyroxine, 2 injections of T.S.H. 30μC I^{131} for 55 mins. (*a*) phase contrast, (*b*) same field, no phase contrast.



(*a*) (*b*)

Figure 12. (*a*) Histological Section. A colloid adenoma (below) and a solid cellular adenoma with a few small scattered acins (upper right).
(*b*) Autoradiography. The colloid adenoma with large distended acins and flat epithelium is looked upon as being in a resting or overripe stage of development.
(From DOBYNS and LENNON, *J. Clin. Endocrinology*, 1948, 8, p. 732).

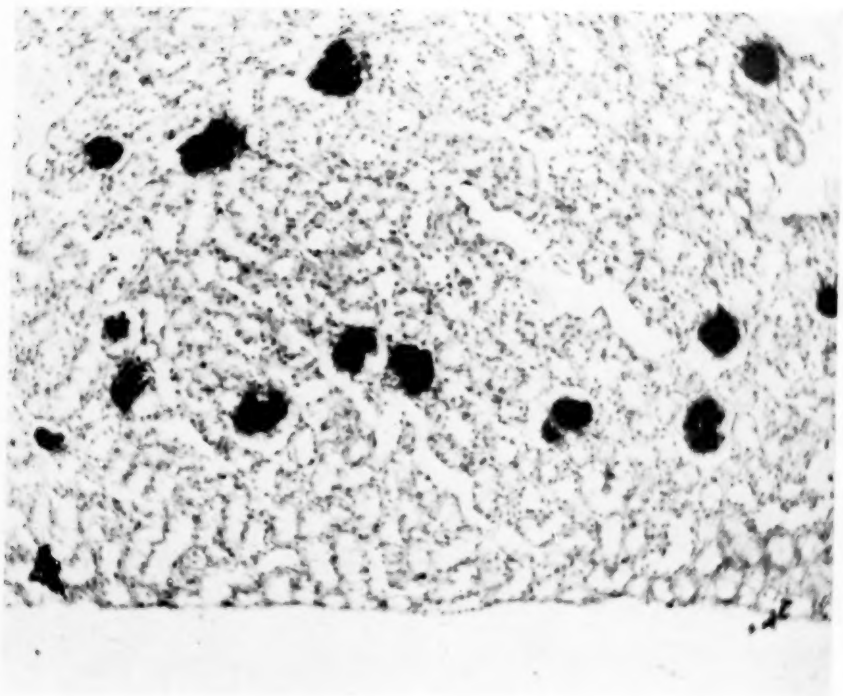


Figure 13. Autoradiograph of section of kidney of mouse injected with S^{35} labelled globulin fraction of anti-mouse-kidney serum showing localization of the anti-serum to the glomeruli. Section stained while in position on the radiographic plate. (From PRESSMAN *et al.*[75]).

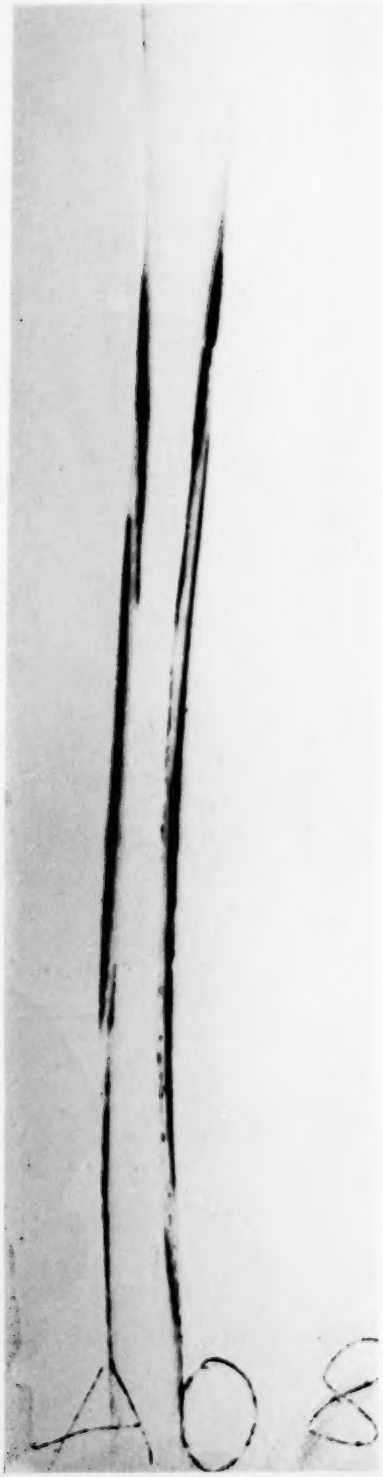


Figure 14. Tibia from rabbit killed 8 weeks after intravenous injection of 150 μC Sr^{90}. Age at time of injection 5-7 weeks. Note (*i*) diagonal lines of localized reaction originally in region of metaphyseal endosteum displaced by growth in length of bone towards middle of the shaft. (*ii*) slightly bowed line of localized reaction with internal diffuse reaction originally in the region of periosteum displaced towards centre of corticalis by growth in width of bone. (*iii*) absence of reaction in either metaphysis. (*Courtesy of* Dr. JANET VAUGHAN).

by so-called physical development. The sensitivity is at present very low.

FINK[35] obtained improved resolving power by expanding the tissue through application of high pressure before autoradiography.

A different approach to autoradiography is the focussing of the emitted electrons in an electron microscope. MARTON and ABELSON[36] achieved a resolving power of about 30 μ using the 83 keV internal conversion electrons from Ga^{67}. BARBER *et al.*[37] obtained 6 μ using the 148 keV, F-line of Th.B. The effective aperture was about 10^{-4} of the complete sphere as compared with 0.3—0.5 for contrast autoradiography. The concentration of radioactive material has to be excessively high because only a small solid angle of a narrow range of β-energy is utilized in these machines.

BIOLOGICAL AND MEDICAL APPLICATIONS OF AUTORADIOGRAPHY

THYROID GLAND

Introduction

There have been numerous autoradiographic studies of the thyroid in experimental animals and humans, since HAMILTON and his collaborators[38] in 1940 and GORBMAN and EVANS[39] in 1941 first demonstrated that radioactive iodine is rapidly incorporated in varying quantities into the colloid masses of the thyroid. By use of their coated autograph technique LEBLOND and GROSS[40] in particular and their colleagues have made considerable advances in the 'morphology of iodine uptake' in experimental animals under varied conditions. Foremost among numerous clinical applications is the work of DOBYNS[41,42,43] and his colleagues who have made extensive use of autoradiographs in their study of the iodine uptake of thyroid nodules and cancers. The simpler techniques of autoradiography are now well established in most institutes where radioactive iodine is in use and they are applied frequently merely to confirm data already given by counting methods carried out on digested tissues. By differential chemical extractions the latter methods do in fact yield more biochemical information. However, the presence of radioactive iodine concentrations in only a few small foci can be detected by autoradiography where it might well be missed by a counting method.

Nature of the autoradiographic material

The avidity of the thyroid gland for iodine, its content and nature, vary with the diet and the state of activity of the gland itself. Up to 10 per cent can be extracted in the euthyroid (normal) subject as inorganic iodide, 90 per cent organically bound, about 60 per cent as diiodotyrosine and 30 per

cent as thyroxine. With present techniques, most if not all of the inorganic iodide is extracted in the processes of fixation and dehydration preparatory to paraffin embedding for the making of sections for autoradiography. The radio-active iodine left in the section has been fixed in a form insoluble in water and in fat solvents and is presumably protein bound[40]. The possibilities that some iodine not yet incorporated into tyrosine is nevertheless fixed or that some free diiodotyrosine and thyroxine are extracted cannot be ruled out. It is currently thought that tyrosine is probably iodinated and then coupled to form thyroxine while still bound by peptide linkage as a part of thyroid protein[44]. Freely diffusible thyroxine is liberated from the thyroglobulin by local proteolytic enzymes under the influence of pituitary thyrotrophic hormone[45]. On these grounds one can assume that thyroid autoradiographs represent protein-bound iodine and do not allow of differentiation between protein-bound diiodotyrosine and thyroxine. It is also generally assumed that there is no significant interchange between atoms of inorganic and organically bound iodine in the thyroid. These two assumptions are supported by the finding that the thyroids of rats given a large dose of methylthiouracil and a tracer dose of I^{131} show no autoradiograph 2 hours later[46]. The thiouracil inhibits organic binding of iodine but not concentration of inorganic iodine[47]. Were there any significant interchange of iodine atoms between the I^{131} and the I^{127} of the previously formed protein-bound iodine in the colloid, these glands should have given a positive autoradiograph in spite of the thiouracil treatment.

Morphology of I^{131} *uptake and relation to thyroid hormone synthesis*

Thyroid autoradiographs illustrated in the literature in general have shown a varied picture. The majority, made some hours or more after administration of the I^{131}, both in animals and humans, show localization of the protein bound iodine to the colloid appearing as round dark blobs which vary markedly in intensity. In autoradiographs made after half an hour in rats[1] and after 5 days in two cases of hypothyroid children[38] there were in addition to blobs many follicles which showed black rings overlying the thyroid cells, and little uptake in the colloid. (Examples of thyroid autoradiographs are illustrated in Figures 7, 10 and 11). The site of uptake of I^{131} in the rat's thyroid and the effects of varying the gland's activity and avidity for iodine were carefully studied by LEBLOND and GROSS[48]. They found that in general there was a variation in rate of fixation of iodine by the follicles, the less active ones being located in the gland periphery, but that all follicles in any gland show some activity at all times. In rats pretreated for 6 weeks with daily injections of 22 μg iodine, a tracer dose of I^{131} was taken up chiefly by the follicular epithelium after 1 hour but appeared almost entirely in the colloid after 24 hours. Rats on a low iodine diet showed I^{131} uptake consistently in the colloid only, both

after 1 and 24 hours. One such rat sacrificed as early as 2 minutes showed a weakly positive autoradiograph over the colloid, but not the cells, of all follicles. Autoradiographs of hypophysectomized rats made after 1 hour showed the I^{131} mostly present in the cells; after 24 hours, both in the cells and in the colloid of some follicles. By the use of an especially thin coating emulsion LEBLOND and GROSS[48] were able to localize the intracellular radioactivity, in the iodine pretreated and hypophysectomized animals, to the apical portions of the cells. (See Figure 4). They put forward these findings as evidence that in the normal formation of thyroid hormone, iodine is first incorporated into protein in the apex of the thyroid cell and this newly formed thyroglobulin is incorporated into the thyroid colloid by diffusion into the follicle lumen. The process is extremely rapid in the stimulated glands of rats on a low iodine uptake (too rapid for autographic visualisation of the intracellular protein binding) and is slowed down in the functionally depressed gland following hypophysectomy.

Our findings[46] are at variance with those of GROSS and LEBLOND[1] who found occasional rings in normal rats. We have always found the iodine incorporated into the colloid and never in the cells, from 5 minutes onwards. The difference is possibly due to a smaller iodine or thyroxine content of our own rats' stock diet. By pretreating 2 rats with daily injections for 6 weeks of 20 μg *dl*-thyroxine we damped down their thyroid activity and obtained rings similar to those found by LEBLOND and GROSS after hypophysectomy (Figure 11). The appearance of protein-bound iodine in the middle of the colloid of active follicles within two minutes of I^{131} administration and its absence from the cells are very significant. Though this picture might result as LEBLOND and GROSS suggest from an extraordinarily rapid intracellular formation and then discharge of thyroglobulin into the colloid we suggest an alternative hypothesis : that in these follicles the iodine diffuses through the follicular epithelium and is bound immediately to the protein in the colloid within the follicle lumen rather than in the cell apices. The iodination of the protein and eventual formation of thyroxine-thyroglobulin presumably depend upon the iodine, follicular cell enzymes and pituitary thyrotrophic hormone present in the follicle. Thus it is conceivable that the ring and blob autoradiographs represent two different types of hormone synthesis in the hypoactive and active follicles respectively. The nature of the material which gives rise to ring autoradiographs merits further study, especially since MORTON *et al.*[49] found that radioactive iodine is incorporated only into the diiodotyrosine and not the thyroxine of the hypophysectomized rat's thyroid.

Miscellaneous experimental applications

The autoradiographic detection of concentration and organic binding of

I^{131} has been used in experimental biology as a marker for the presence of active thyroid tissue. GORBMAN[1] studied the evolution and embryonic development of the thyroid. Starting with higher invertebrates he found organically bound I^{131} in the stolon, a structure arising from the pharynx of the Ascidian *Perphora annecteus* Ritter. He showed in lamprey larva that the iodine was taken up by the endostyle, a pharyngeal derivative, the precursor of the adult lamprey's thyroid gland. Tadpole thyroids fixed I^{131} only when the animals' total length exceeded 10 mm, which correlated directly with the stage of first development of colloid containing follicles. This same correlation applied to rat foetuses whose thyroid glands first show differentiation of solid cords into colloid containing follicles at the 19th day of development, the first day on which thyroid autoradiographs gave a significant blackening. Thus, GORBMAN has elegantly confirmed the entodermal pharyngeal derivation of the thyroid and its activity in the embryo coincident with colloid formation. CHAPMAN *et al.*[50] found that uptake of iodine by the human foetal thyroid was first demonstrable at 14 weeks, also coincident with the first signs of colloid formation.

Autoradiography has been used to study the degree of destruction and amount of recovery in the thyroids of animals given large doses of radioactive iodine. Thus, FINDLAY and LEBLOND[51] found that function was destroyed first in the more active central follicles of the rat's thyroid. SPEERT *et al.*[52] injected pregnant mice with 200 μc I^{131} and found the maternal glands similarly damaged. Surviving offspring whose mothers had been injected from the 17th day of pregnancy onwards were retarded in growth. Autoradiographs of their thyroids in later life showed I^{131} uptake by regenerating hyperplastic follicles. GORBMAN[53] found a slight but definite I^{131} uptake into the colloid of inactive thyroid follicles which had survived a large radiotoxic dose of I^{131} in mice.

BENNETT and GORBMAN[54] have studied the function of mouse thyroid transplanted into hosts of the same strain. Autoradiographs showed the greatest depression of function to occur on the third day when cellular and vascular recovery were at their height. Function appeared restored to normal by the 10th day. The ability of induced thyroid adenomas in the rat to bind I^{131} was demonstrated by DONIACH[55].

Clinical applications

Autoradiographs of human thyroid tissue removed at operation have proved invaluable in the correlation of localized iodine uptake with histology, with clinical findings and with results of pre-operative scanning of the neck for distribution of radioactivity. In addition, autoradiographs show whether a thyroid cancer takes up iodine and is thereby amenable to treatment with therapeutic doses of I^{131}. They also help to demonstrate

whether total thyroidectomy and prolonged thiouracil treatment lead to an increased avidity of metastases for iodine. The most fruitful advance in the correlation of structure with function have come from the study of nodular goitres and thyroid cancers. There have been numerous reports of findings in individual cases or small groups of patients over the past few years and in addition excellent summaries of the findings of impressive series of cases both at the *Massachusetts General Hospital, Boston*[41,43] and the *Memorial Hospital, New York*[56].

DOBYNS and LENNON[41] made numerous autoradiographs of large blocks which included both nodules and adjacent thyroid tissue; the former were called 'hot' or 'cold' nodules according to their uptake respectively of more or less I^{131} than the adjacent follicles (Figure 12). Hot nodules were sometimes but not necessarily associated with thyrotoxicosis; they are mostly composed of quite tall cells relatively uniform in height. Histologically hyperplastic cold nodules showed considerable variation in cell height and often a papillary structure. Colloid nodules lined by a flat epithelium were also relatively non-functioning. Apart from these last two types; the nodules in general showed a good correlation of degree of differentiation with intensity of iodine uptake. Thus the least differentiated most embryonic types took up very little iodine in contrast to the well marked activity of the well differentiated follicular nodules. With this technique, DOBYNS and LENNON were also able to differentiate in cases of toxic nodular goitre whether the Graves' disease was due to one or more hot nodules or else to excessive activity of diffuse extranodular thyroidal tissue associated with incidental cold nodules. Their method is particularly helpful in that it always allows a comparison to be made between any nodule and its neighbouring 'normal' tissue. By correlation of all their data, they were able in time[42] to make a shrewd preoperative assessment of the type of goitre and the nature of individual nodules from the results of mapping of distribution of radioactivity in the neck. Their studies of the correlation of the macroscopic appearance of thyroid tissue and nodules with autoradiographic properties often proved of considerable help at operation.

In a series of autoradiographs of large sections of operation specimens of simple goitre, TAYLOR[57] found that iodine was concentrated intensely in a few discrete areas, rather than diffusely as in the normal thyroid. Further analysis showed that these active areas were made up of fairly uniform follicles of a mean diameter of one half to one third of that of the surrounding slightly active ones, and were lined by a taller and more uniform epithelium.

FITZGERALD and FOOTE[56] summarize their findings in 100 consecutive cases of carcinoma of the thyroid. Forty-six gave autoradiographic evidence of concentration of I^{131} in the carcinoma, but in every case, less than that in adjacent thyroid tissue. Of 258 separate lesions studied, 26 per cent of

papillary, 77 per cent of alveolar and follicular, 39 per cent of solid and 16 per cent of Hürthle cell carcinomas concentrated I^{131}. None of the anaplastic, giant and spindle cell carcinomas took up any iodine. In nearly all the positive specimens, iodine uptake was restricted to colloid containing follicles whereas areas of pure papillary or solid carcinoma did not concentrate iodine. On the other hand by no means all of the neoplastic colloid containing follicles showed an avidity for iodine. Their findings emphasize the need for the techniques now being applied[43] which attempt to increase the differentiation and iodine uptake of metastases of thyroid cancer.

HORMONES

The chief studies to date on the localization of hormones have been made with radioactive thyroxine. There are a few recent reports on the use of labelled oestrogens and anterior pituitary extracts. Hormones have also been studied by their effect upon the incorporation of a radioactive element into target organs, as for example the effect of thyrotrophic hormone upon the uptake of radioactive iodine in the thyroid gland[58].

Thyroid hormone

The peripheral distribution and metabolic fate of thyroid hormone have been studied chiefly by chemical methods to which counting techniques have been added since labelled thyroxine has become available. Autoradiography has helped in the localization of radioactive thyroxine made either by the animal's own thyroid gland following an injection of I^{131} or else synthesized in vitro. GROSS *et al.*[2] found a high concentration of radioactivity due to natural thyroid hormone distributed throughout the liver and cortex of the kidney and a lesser diffuse reaction throughout the heart, spleen and pancreas, while in the adrenal the concentration was greater in the cortex than the medulla. In view of the established reciprocal relation between thyrotrophic hormone output by the anterior pituitary and thyroxine output by the thyroid gland[59] attempts have been made to localize thyroxine in the pituitary. JENSEN *et al.*[61] killed rabbits 4 hours after an intravenous injection of 1 mc I^{131} and obtained no autoradiographs of the pituitary or neurohypophysis. On the other hand rabbits killed 4 hours after receiving 3 mg of synthetic radioactive *l* thyroxine labelled with I^{131} showed strong autoradiographs of the medial eminence of the tuber cinerum, neural lobe of the pituitary and infundibulum. The pars anterior of the pituitary gave a very slight autoradiograph. This strong affinity of the neurohypophysis for thyroxine suggested to the authors that it might be the site of regulation of output of thyrotrophic hormone.

Anterior pituitary preparations

SONENBERG *et al.*[62] labelled a preparation rich in adrenocorticotrophic

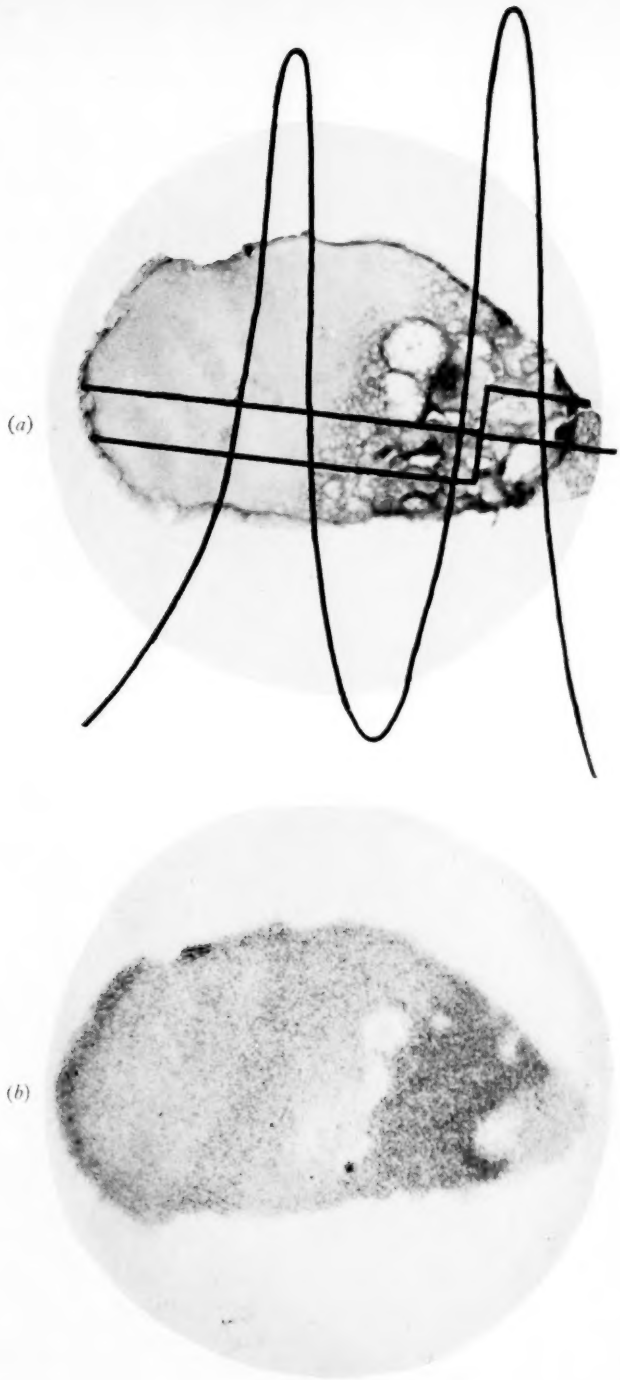


Figure 15.

(*a*) 10μ erythrosin-stained section of amoeba 288 hr after ingestion of ciliates which have metabolised C^{14}. The amoeba was centrifuged about 30 mins at 1000 × G before fixation. Heavy pole to the right. The graph represents grain density in the rectangular areas outlined.

(*b*) The restripped autoradiograph of the section shown in *a* on which the grain counts were made.

(From ANDRESEN *et al.*[116])

activity with tracer I^{131}, which was then injected intracardially into rats. These were sacrificed at varying intervals and their organs assayed for radioactivity. Only the adrenals and thyroids gave positive autoradiographs. The adrenals showed a selectively stronger autoradiograph over the inner layers of the cortex; the material appeared in the adrenals immediately after injection and had practically disappeared by 2 hours. Adrenal autoradiographs were not obtained in control rats injected with iodinated bovine serum albumin labelled with I^{131}.

SONENBERG *et al.*[63] carried out similar studies with I^{131} labelled prolactin. No significant radioactivity was detected in resting or lactating mammary glands, but the ovaries showed a significant concentration reaching a maximum in 30 minutes, shown by autoradiography to be in the corpora lutea, with essentially none in the ovarian follicles.

ULRICH *et al.*[64] injected calcium chloride tagged with radioactive Ca^{45} intravenously into rats hypophysectomised 90 days previously, a number of which had received daily injections of pure anterior pituitary growth hormone during the previous 3 weeks. They were killed two hours after receiving the calcium. Autoradiographs of the rats' tibias showed a dense line of deposited Ca^{45} in the proximal epiphyseal plate region only in those animals which had received growth hormone.

Oestrogens

There have been a number of reports on experiments carried out with radioactive oestrogenic compounds variously labelled with I^{131} or S^{35} or Br^{82} using a counting assay technique[65]. The solubility of these compounds in fat solvents renders them awkward for autoradiographic study and to date there is only one recorded autoradiograph localizing an oestrogen. This was made on a frozen section of mouse skin, the oestrogen (non-biologically potent iodinated α oestradiol) being concentrated in the fibrous tissue of the derma[66].

ANTIGEN ANTIBODY REACTIONS

Both protein antigens and antibodies have been labelled with a radioactive tracer, usually I^{131}, and localization of reaction in the body ascertained by counting techniques or autoradiography. This advance has coincided with the development of nonradioactive dye methods of labelling protein antibodies[67,68,69] including fluorescent dyes.

WARREN and DIXON[70] sensitized guinea pigs to a partially iodinated bovine gamma globulin and determined the distribution of radioactivity in the tissues of animals killed by anaphylactic shock induced within 5 to 8 minutes by a minimum lethal dose of I^{131} tracer labelled iodinated globulin. Autoradiographs showed that outside the contents of blood-vessels, the heaviest concentration of radioactivity was in perivascular and peri-

bronchial oedematous congested fibrous tissue and diffusely through the liver. There was no significant activity in the bronchial muscle or mucosa or other organs. This pulmonary concentration was observed only in sensitized animals and not in controls. The liver appeared the same in the controls as in the sensitized animals. These findings seem to show the distribution of labelled plasma, rather than the site of antigen antibody reaction, in the anaphylactic guinea pig. In later work, however, DIXON and WARREN[71] confirmed the specificity of the pulmonary concentration of antigen by the ingenious experiment of injecting labelled *albumin* into guinea pigs rendered sensitive and then anaphylactic with *fibrinogen*. The albumin showed no concentration in the oedematous bronchial walls, though it did so in another group of *albumin* sensitized and shocked animals.

PRESSMAN *et al.*[72] radioiodinated the globulin fraction of both anti-rat and anti-mouse kidney serum, confirmed the specificity of the material and made autoradiographs of the kidneys of animals killed 5 days after intravenous injection with these antibodies. They showed clear cut concentration of the antibody restricted to the glomeruli. Measurement of the radioactivity of various digested tissues had shown a significant concentration in the kidneys, not found in assays of kidneys of control animals injected with other labelled sera[73]. EISEN *et al.*[74] later obtained positive renal glomerular autoradiographs with rat anti-lung serum and postulated common antigens in the two organs responsible for the development of kidney localizing antibody. PRESSMAN *et al.*[75] also obtained excellent autoradiographs of mouse renal glomeruli in animals injected with specific antibodies labelled by coupling with diazotized aminobenzenesulphonic acid containing radioactive S^{35}, Figure 13. These studies show that autoradiography can be of great use in research with labelled proteins. At the same time it has been established that some proteins can be labelled effectively with one or two substituent groups per protein molecule with only slight loss of immunochemical reactivity[76].

TUMOURS

The selective uptake of tissues of radioactive isotopes in the form of elements or labelled compounds might clearly prove very useful in diagnostic and therapeutic application. Studies to this end have been carried out on the uptake of specific normal tissues as well as tumours, using mostly counting techniques. With the exception of the thyroid gland, autoradiography has so far been surprisingly infrequently applied. Phosphorus is taken up by most tissues, but preferentially by calcified and by rapidly growing ones. LAWRENCE[110] made autoradiographs of whole mice bearing lymphosarcomata which showed uptake of P^{32} into the tumours after administration of sodium radiophosphate. STEINBERG and SELVERSTONE[77] studied 18 mixed primary and secondary brain tumours

in patients injected with 0·5 to 4 mc of carrier free P^{32} 12 to 72 hours before craniotomy. Contact autoradiographs made on thick frozen sections of material obtained at operation or autopsy showed selective uptake of P^{32} by viable tumour in contrast to normal brain and necrotic tumour. Colloidal preparations of chromic radiophosphate[78], uranium oxide[79] and gold[80] are taken up by the cells of the reticuloendothelial system and have therefore been used in the treatment of malignant lymphomas. Autoradiographic localization of various isotopes in tumours have been demonstrated with Sr^{89} in osteogenic sarcomas[81], Ga^{72} in experimental osteogenic tumours[82], Au^{198} retained in experimental tumours into which it had been directly injected[80], and Zn^{65} in serosal metastases after intraperitoneal injection[83]. SKIPPER *et al.*[84] demonstrated autoradiographically the rapid incorporation of sodium formate labelled with C^{14} into mouse leukaemic cells. So far autoradiographs do not appear to have been used in a number of reports describing the distribution in treated animals of radioactively labelled chemical carcinogens.

BONES AND TEETH

Heavy elements

One of the first uses made of the autoradiograph method was in the study of the distribution of heavy radioelements in bone. They include radium, uranium, cerium, americium, plutonium, curium, lanthanum, strontium and yttrium. These elements are foreign to living organisms, and some are produced by nuclear reactions in atomic piles. They are all deposited in bone, usually irreversibly, and a knowledge of their distribution within this tissue was necessary for the control and prevention of radiation damage. This work is reviewed by HELLER[3] and GROSS *et al.*[2]; the reader is also referred to HELLER[85] for a description of the autoradiographic pictures obtained.

Radiophosphorus

A second field of enquiry is concerned with the growth and metabolism of bone using P^{32}. In 1946 BÉLANGER and LEBLOND[27] published an account of their newly-invented coating technique, together with a brief announcement of the results of its application to the study of the deposition of phosphorus in the bones of newborn animals. A fuller account of these experiments appeared in 1948[86] and 1950[87] and is summarized by GROSS *et al*[2]. P^{32} was injected subcutaneously into newborn rats and kittens: it was shown by autoradiographs, in conjunction with counts of the specific activity of blood and bones at intervals after injection, that phosphorus enters the bones and teeth with very great rapidity, being appreciable after 1 hour and maximal at 4 hours. The amount deposited does not alter

appreciably between 4 and 72 hours after injection, indicating that an equilibrium between blood and bone phosphate is maintained. The authors conclude from this distribution of deposited P^{32} that there is no 'stable' bone in the newborn animal but that all bony structures are in equilibrium with the phosphorus pool. With the growth of the animal, areas appear which show a relatively weak and diffuse reaction if the autoradiograph is taken a few hours after injection, when the blood level has dropped. Since the amount of activity in these areas decreases with time after injection, the presence of P^{32} in them is interpreted as being due to ionic exchange, and they are considered to be regions of 'stable' bone. The P^{32} laid down during growth, however, remains in the layer in which it was deposited, presumably as insoluble crystalline salts in which ionic exchange is low. By following the movement of these layers of deposited P^{32} LEBLOND and his colleagues have built up a picture of the dynamics of bone growth[87].

The growth of teeth has been followed in a similar way by BÉLANGER and his colleagues[88,89] using injected radiophosphate in hamsters and mice. Here again two types of autoradiographic picture appear : a diffuse reaction which decreases rapidly with time after injection, and a persistent localized picture which is thought to result from the deposition of P^{32} during growth, Figure 5. By combining the autoradiograph method with MALLORY'S anilin blue-acid fuchsin stain, BÉLANGER[89] has shown that the distribution of P^{32} is correlated with blue-staining areas of the enamel *i.e.* that only enamel at a certain stage of maturity incorporates P^{32} under the circumstances of his experiment. In the dentine P^{32} is laid down in relation to mineralization, in a region near the odontoblast layer, but recent work[89] suggests that the process is not one of simple appositional growth, and the presence of two insoluble salts, one more labile than the other, is suspected.

Radiostrontium and radioyttrium

New work on the distribution of radiostrontium and radioyttrium in rabbit bones has been carried out by VAUGHAN and her colleagues[90,91,92]. This work was stimulated by the possible radiation hazard due to these isotopes, but some facts of fundamental interest have emerged from it. Sr^{89} was injected intravenously into rabbits as chloride (with added carrier) and sections prepared of the long bones of the limbs, Figure 14. Satisfactory autoradiographs were made with the stripping film method, although the thickness of sections which can be made of undecalcified bone limits the resolution. Autoradiographs show that in young animals there is considerable concentration of strontium in sites of active bone growth *i.e.* beneath the epiphyseal plate, in the region of the endosteum, in the metaphyseal part of the shaft and in the region of the periosteum in the middle of the

shaft. These localized reactions move with time, their rate of movement depending upon the age of the animal. The autoradiograph picture is similar to that obtained with P^{32} by LEBLOND *et al.*[87] in rats, and it is suggested[92] that radiostrontium is incorporated by coprecipitation with the bone salt as strontium phosphate in sites of active calcification. The incorporation is most marked during the time at which the blood level is high : separate experiments show that the blood level of Sr^{89} drops rapidly during the first 3 hours after injection.

In older animals, in which the epiphyseal plate has closed and periosteal and endosteal growth has ceased, there is no definite localization of strontium.

A diffuse distribution is observed throughout mineralized regions of the bones of all treated animals, in addition to the localized reaction seen in young bones. Such a diffuse autoradiograph, when obtained in bone with P^{32} by LEBLOND and his colleagues (see GROSS *et al.*[2] for refs.) was interpreted by them to mean that labile P was present throughout the formed bone crystal. VAUGHAN and her colleagues disagree that this is necessarily implied by such pictures. They point out that if the radioactive atom, whether phosphorus or strontium, is adsorbed by ionic exchange on the surface of the bone crystal, it will diffuse into the crystal and will then not be liable to desorption. They interpret the non-localized presence of Sr^{89} throughout bone as due to exchange, diffusion, and subsequent retention, and suggest that the diffuse distribution found in areas adjacent to those in which radioactive isotopes are deposited during growth may have the same origin.

VAUGHAN *et al.*[90] find that radioyttrium is localized in bone in a pattern different from that of radiostrontium. This can be observed when the two isotopes are injected separately and when Sr^{89} is chosen in preference to Sr^{90} since the latter breaks down into Y^{90} : Y^{91} when injected into rabbits is laid down around the Haversian canals, and near the epiphyses and beneath the periosteum. The picture is similar to that shown by SCOTT *et al.*[93] for cerium, which was thought to have been deposited in relation to blood vessels.

LOCALIZATION OF ISOTOPES WITHIN CELLS

The first application of the autoradiograph method to the localization of an isotope within a cell is that of MULLINS[94]. He chose the long cells (40 mm) of *Chara* and *Nitella*, one end of the cell being placed for a time in radiophosphate solution. By virtue of the great length of the cells, contact autoradiographs were adequate to resolve discrete concentrations of activity along them.

In 1950 PELC and SPEAR[95] described work in which chick fibroblasts in tissue culture were fed with P^{32} as phosphate and cells from subsequent

explant generations autoradiographed by the stripping film method. On thin single-cell layers this method gave a resolving power of about 2 μ. After alcohol fixation and water washing, the P^{32} was present mainly in the cytoplasm, with less in the nuclei and vacuoles and none in the nucleoli. Grain counts provided a quantitative estimate of the relative amounts of P^{32} in the different cell parts. It is likely that this was present in nucleic acids : its absence in the nucleolus is surprising.

Autoradiography has been used in the localization of tritium in yeast[96]. This work has not as yet produced many results of biological significance. A differential distribution of P^{32} in the food vacuoles of *Paramecium*[97], Figure 9, has been demonstrated, using track autoradiography, with a resolving power of about 5μ. KING *et al.*[97] have observed that the number of tracks per organism in *Paramecium aurelia* grown in radiophosphate shows a skew distribution, so that some individuals take up more P^{32} than expected. This is not obviously related to the division cycle, and its explanation is unknown.

The first autoradiographs of chromosomes (mouse meiotic prophases) were published by HOWARD and PELC[98]. The same authors have demonstrated that P^{32} present in DNA in bean root cells is localized in the nuclei of resting cells and the chromosomes of dividing ones. They have also[99,100] described the distribution of S^{35} along a chromosome. This is discussed below.

LABELLING OF CELLS WITH P^{32} AND S^{35}

The stability of phosphorus in the desoxyribonucleic acid (DNA) molecule, and the fact that this compound is retained in the cell during histological processing, has made it possible to label nuclei and to follow them in time and in space. This method was used by LEBLOND[101] in demonstrating the movement of cells in the epithelium of the intestinal villi of the rat. Two hours after the injection of radiophosphate, activity was found in the crypts of Lieberkühn, where mitotic activity was high : after 24 hours the labelled cells were observed part way up the sides of the villi.

A similar method was used by HOWARD and PELC[98] in studying the duration of stages of spermatogenesis in the mouse. By sacrificing animals at intervals after injection of radiophosphate and studying autoradiographs of squash preparations of the testis, in which single cells could be distinguished, it was possible to show that nuclei incorporate P^{32} in acid-insoluble form at certain stages only of the spermatogenic process (the spermatogonia, and a period during the first meiotic prophase). Thus cells could be labelled with reference to the time at which the P^{32} was given. The form in which the observed P^{32} had been incorporated was not determined but it is probable that in nuclei it was mainly present as DNA.

KUPER and PELC[102] have recently succeeded in labelling with P^{32}, probably in DNA, the oocysts of *Plasmodium gallinacium* (fowl malaria). The method was to feed P^{32} to an infected mosquito and examine sections of the midgut after an appropriate interval. Such sections showed radioactivity in the oocysts, and autoradiographs were obtained also by feeding S^{35} as sulphate to the hosts, showing that the parasites metabolize sulphate ion.

DISTRIBUTION OF P^{32} IN RABBIT OVARIES

ODEBLAD[103] has described autoradiographs of ovaries of rabbits treated with inorganic orthophosphate. The form of the P^{32} in the processed sections is thought to be mainly DNA and phosphoprotein. 2-24 hours after injection the ova were found to contain very little P^{32} while the granulosa and theca interna showed strong uptake. Corpora lutea take up considerable amounts during development but very little during regression. The autoradiographs were made by contact with x-ray film and had a resolving power of 18 μ at best. An attempt at estimating relative amounts of P^{32} present in lipid and acid-insoluble compounds of different tissues of the ovary was made by grain counting and by microphotometry.

DISTRIBUTION OF FE^{59}, CU^{64} AND BA^{140} IN LARVAE OF DROSOPHILA

POULSON and BOWEN[111] have used autoradiographs (floated section technique) to supplement their histochemical studies of the distribution of metals in the larvae of *Drosophila* species. In some tissues Fe^{59} was found to become concentrated in the cell nuclei, the amount relative to that in the cytoplasm increasing with time. This iron is apparently in equilibrium with the iron stores of the body, since it can be flushed out by feeding for a time on food rich in stable iron. It is in a form not soluble in neutral alcohol-formalin and other fluids used in histological processing. It is considered to be present, at least in part, as catalase and cytochrome C.

Cu^{64} did not become concentrated in the cell nuclei in any tissue. Further autoradiographic work is expected to yield more precise information on the intracellular localization of copper. Meanwhile, contact autoradiographs have been used[112] to study the movement of Cu^{64} in dissected guts of larvae of *Drosophila* species.

BOWEN[113], in studying the uptake of barium in larvae of *Drosophila repleta*, has found, using Ba^{140} and autoradiographs, that barium may be retained for 10-20 hours in the anterior mid-gut between the peritrophic membrane and the gut wall. Here it is absorbed, and is rapidly transferred to the anterior malpighian tubules, where it is stored until after pupation. Some is also found in scattered patches of cells of the posterior mid-gut.

ABSORPTION OF LABELLED ANTIBIOTIC BY SENSITIVE BACTERIA

LACASSAGNE *et al.*[114] have used autoradiographs to provide a striking demonstration of the different behaviour of sensitive and resistant bacteria with regard to the absorption of an antibiotic. A preparation of 2 — sulfanilamidopyridine labelled with S^{35} was applied to cultures of *Staphylococcus aureus*, *Streptococcus haemolyticus* and *Escherichia coli*. After 24 hours the cells were washed, spread on a slide and autoradiographed. It was found that the first two species, which are sensitive to sulphonamides, gave autoradiographs while *E. coli*, which is resistant, did not. This suggests that the resistance of bacteria to antibiotics is due to their non-absorption rather than to intracellular neutralization of their effects.

STUDIES OF THE SYNTHESIS OF DNA AND NUCLEOPROTEIN

It has been found that if seedlings are given radiophosphate for a period of time before fixation, the P^{32} seen in an autoradiograph after hot HCl extraction of the fixed root tissue is present as desoxyribonucleic acid (DNA) phosphorus. HOWARD and PELC[8,104] have used this fact to study the time of synthesis of DNA in the cell cycle and the influence on it of X-radiation. Using the root meristem and the neighbouring differentiating tissue of *Vicia faba*, they find that (1) DNA is synthesized in the early part of the interphase; during the last third of the interphase and during mitosis no synthesis is observed. (2) In the meristematic region of the root, synthesis occurs only in cells which are preparing for division, and not in those which will differentiate without further mitosis. (3) The absence of autoradiograph above the nuclei of cells which had begun to differentiate before P^{32} was given indicates that extremely little exchange of phosphorus occurs between the DNA molecule and other phosphorus in the cell. (4) X-radiation reduces markedly the number of cells which synthesize DNA in a given period, and also the amount synthesized by the remainder. Some of these conclusions are in agreement with other histochemical observations carried out with staining techniques and with photometric measurements at the nucleic acid-absorption wave-length in the ultraviolet. It is to be expected that since DNA is an essential component of the nucleus, its amount must increase with nuclear reproduction; SWIFT[105] has shown in maize roots that the amount of Feulgen-staining material doubles before the beginning of prophase : LISON and PASTEELS[106] using sea-urchin eggs, observe it to increase in telophase in preparation for the next division. Further, it has been shown by HEVESY[107] and by HOLMES[108] that in the intact animal the amount of DNA synthesized drops markedly after exposure to whole-body X-radiation; and biochemical work

has revealed the great stability of the DNA molecule. The autoradiograph technique promises to be particularly valuable in conjunction with other methods of studying the metabolism of DNA since it permits an examination of the behaviour of DNA phosphorus, and of other constituents with labelled precursors, while the Feulgen method examines the sugar, U-V absorption the base, and methyl green staining reveals polymerization. A combination of methods should yield much new information.

If S^{35} is given to roots as sulphate, radioactivity is found, after hot HCl extraction, above a percentage of nuclei. This proportion of cells is similar to the proportion which synthesizes DNA during the same period, and the suggestion has been made[109] that the S^{35} is incorporated in nucleoproteins which are built up at the same time as DNA. X-radiation does not apparently affect the number of nuclei which incorporate S^{35}. The resolving power is sufficient to allow the distribution of S^{35} along chromosomes to be examined, Figure 6, and when this is done for a particular chromosome pair (the 'M' of *Vicia faba*), the distribution is found to show a constant and characteristic pattern in each arm[99,100]. This is taken to indicate that different kinds of sulphur protein, or different amounts of sulphur protein, are synthesized in different regions of the chromosome, and it is suggested that this phenomenon may be genic in origin.

METABOLISM OF CARBON IN AMOEBA

The stripping film technique has been applied by ANDRESEN, CHAPMAN-ANDRESEN and HOLTER[116] to the study of C^{14} metabolism in amoebae. Organisms were fed on ciliates (*e.g. Paramecium bursaria*) whose symbiotic algae had metabolised C^{14} from labelled carbonate in the medium. The amoebae were fixed after suitable periods and sections were autoradiographed. After exposure the film was restripped and mounted separately, allowing the sections to be stained and examined cytologically. If before fixation the amoeba is centrifuged so that the cell contents are stratified into layers, the distribution of C^{14} amongst cytoplasmic inclusions having different specific gravities can be observed. This was done quantitatively by counting the number of developed photographic grains per unit area in a strip through the length of the section. The results show that up to about 24 hours after the active meal has been taken, activity is largely concentrated at the heavy pole of the amoeba. Between 24 and 400 hours a band of activity appears also in the lighter half. In later stages of digestion (up to 900 hours) this second peak disappears while the concentration in the heavy part remains (Figure 15). The chemical composition of the compounds containing C^{14} after digestion is unknown, and it is not possible to say, on the basis of autoradiographs, whether the active compounds are bound to mitochondria (which are present in the active layer

of the lighter half) or other granules, or are part of the cytoplasmic ground substance. It is clear, however, that the digested C^{14} labelled food is not accumulated in specific bodies, as has been suggested by MAST and DOYLE[117].

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POLARISED ULTRAVIOLET MICROSPECTROGRAPHY AND MOLECULAR STRUCTURE

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INTRODUCTION

THE present review deals with the application of the ultraviolet absorption spectra of molecules containing conjugated ring systems (*e.g.* purines, pyrimidines, benzenes *etc.*) to the problems of structure analysis of crystals and oriented systems of biological interest and origin. Absorption spectroscopy gives data which may be used in the fields of structural or analytical chemistry, and for many years biologists and biochemists have made use of absorption spectroscopy as a means of identifying the components and breakdown products of biological material. As early as 1883 SORET[1] suggested that the ultraviolet absorption of solutions of proteins was due to the aromatic amino acids contained in the protein, although it was not until about forty years later that this hypothesis was definitely established experimentally[2,3,4]. HOLIDAY[5] has shown that the ultraviolet absorption of a protein in the region λ2700Å—λ3000Å may be considered as due to a two-component mixture of tyrosine and tryptophane, and that the absorption in this region of the spectrum may be used to estimate quantitatively the tyrosine and tryptophane content of the protein. This work, together with the accumulating mass of data on the absorption of biochemical material, was in part responsible for the initiation in Sweden of the work of CASPERSSON, HAMMARSTEN and HAMMARSTEN[6,7] which resulted in the development of microspectrographic techniques to determine the ultraviolet absorption of small areas of biological specimens, either living or cytochemically fixed; and so endeavour to understand the role of nucleic acid in the living cell during cell metabolism and protein synthesis. This work of CASPERSSON and his colleagues has been dealt with at greater length elsewhere[8], but it must be recognised that it has resulted in the development of techniques which may be applied to the structural problems discussed in this review. With the exception of some early measurements by SCHMIDT[9] and SIGNER, CASPERSSON and HAMMARSTEN[10,11] on the visible birefringence and the absorption of polarised ultraviolet radiation by oriented nucleic acid both on purified material and on the thread-like heads of locust sperm, the application of ultraviolet absorption measurements to the structure of biological molecules

has only become important during the post-war years. Considerable work has been carried out on solutions and vapours of substances containing planar, or approximately planar, aromatic and heterocyclic rings, and the interpretation and assignment of the various spectral bands has been made in terms of a valence-bond treatment of the π-electron, or by empirical relations guided by quantum mechanical considerations[12]. However, although this treatment appears quite sound, actual experimental evidence on the absorption spectra of planar ring molecules is very scarce, although some real evidence has been obtained on hexamethyl benzene[13] and on several substituted pyrimidines[14,15,16]. KASTLER, BENEL and BRODERSEN[17,18] have carried out experiments on the fluorescence emission from naphthalene crystals containing anthracene impurity, and some evidence of structure may be obtained from this sort of work; but the theory of the process is still incomplete. Using the data obtained from observations on the planar rings of pyrimidine and benzene, and assuming that other approximately planar conjugated ring systems have similar spectral characteristics, ultraviolet absorption measurements may be used to obtain evidence of the orientation of the absorbing groups in large molecules of biological interest. However, when dealing with absorbing groups whose spectra have not been fully studied, absorption data must be interpreted very cautiously.

Although this review deals primarily with the absorption of polarised ultraviolet radiation by crystals and oriented systems of biological interest, some mention must be made of the work of BRUMBERG and PEKERMAN[19] on the ultraviolet absorption spectra of micro-crystals of zinc sulphide phosphors containing copper and manganese impurity. This work was carried out using a reflecting microscope and spectrographic camera, the specimen being illuminated by the radiation from a krypton arc, and is of interest as it appears to be the first record of the use of this micro-spectrographic technique on micro-crystals.

INTERPRETATION OF ULTRAVIOLET DICHROISM

The phenomenon of dichroism, the preferential absorption of light polarised in a particular direction, was first observed in the mineral tourmaline in 1815 by BIOT. Since then a vast number of organic and inorganic crystals have been discovered which exhibit the phenomen, but at present the theory of the process is inadequate. A linear absorbing group (*e.g.* a linear system of conjugated double bonds) is characterised by two optical densities $d\perp$ and $d\parallel$, perpendicular and parallel to the direction of the absorbing bonds. The difference $d\parallel - d\perp$ is termed the dichroism of the system, while the ratio $d\parallel : d\perp$ is the dichroic ratio. In general, a crystal will have three optical densities corresponding to the principal orthogonal axes of the triaxial absorption ellipsoid. In practice,

however, observations are usually made on crystals of fixed orientation, or on films and oriented sheets; thus two optical densities are obtained for light polarized parallel to the principal extinction directions of the crystal or oriented structure. In calculations involving the estimation of the thickness of a specimen in terms of these optical densities, and the mean optical density d obtained from observations on solutions of known concentration with a spectrophotometer, the following relation is used:

$$d = \frac{1}{3}(d_x + d_y + d_z)$$

where d_x, d_y and d_z are the three principal optical densities of the crystal.

The ultraviolet absorption in the region λ2400A—λ3000A is apparently due to systems containing conjugated double bonds, and in most of the biological material the interesting absorbing groups are usually aromatic and heterocyclic rings (*e.g.* purines, pyrimidines, benzenes). It is not unreasonable to assume that a planar ring compound will behave from the point of view of the absorption of polarised radiation like two linear absorbers perpendicular to one another in the plane of the ring. Thus, it would be expected that radiation polarised with the electric vector perpendicular to the plane of the ring would be transmitted, while radiation polarised parallel to the plane would be preferentially absorbed. By anology with the linear case the ratio $d\parallel : d\perp$ should be large, but as interaction occurs there is no *a priori* reason to assume that $d\perp$ is vanishingly small, or that $d\parallel$ is the same in all directions in a symmetrical ring — *i.e.* dichroism in the plane of the absorbing ring is possible. There is now some definite evidence[13,14,15,16] that these assumptions are reasonable, and the interpretation of dichroism in terms of molecular structure is possible in simple cases.

In general, the absorbing groups in crystals and oriented structures need not lie parallel to each other; hence observed ultraviolet dichroic ratios are often small (1:1 to 1:2). It is of interest to consider what information can be obtained in such cases. It can be assumed that the system is partly disoriented, and the small dichroism is a measure of the degree of orientation; this assumption is somewhat negative and clearly does not apply to purely crystalline material. Alternatively, it may be assumed that the material is fully oriented and that the dichroism is a measure of the mean angle of tilt to the axis of the various absorbing groups in the molecule. In all the results considered in the present review, this latter assumption is made. Assuming that the dichroic ratio for a planar ring is very large, and that dichroism in the plane of the ring is small, the variation of dichroic ratio with angle of tilt for some simple models is easily calculated. Thus, for a uniaxial system with rotational symmetry (*e.g.* a fibre in which the absorbing planar groups are oriented

such that the normal to the plane of the ring lies on a cone of semi angle θ generated about the fibre axis) the dichroic ratio D is given by[20]

$$D_{\text{Fibre}} = \frac{\sin^2\theta}{1 - \frac{1}{2}\sin^2\theta};$$

while in the case of oriented films [15]

$$D_{\text{Sheet I}} = \sin^2\theta/\cos^2\theta$$

if the normal to the plane of the absorbing ring makes an angle θ with the direction of shear, and lies in a plane normal to the direction of incidence of the radiation; and

$$D_{\text{Sheet II}} = \sin^2\theta$$

if the normal to the absorbing groups makes an angle θ with the direction of shear and lies in the plane of incidence of the radiation.

Using these simple relations, some indication of the mean orientation of the ultraviolet absorbing groups may be obtained. In the above considerations it is assumed that only parallel light is passed through the dichroic medium. In practice this is not the case, as in microscopy parallelism and resolution are incompatible, but it is always advisable to use a small condenser aperture (*viz.* 0·3 N.A.). With such a condenser aperture, errors due to the use of a converging beam are in general negligible.

METHODS AND APPARATUS

Microspectrographic apparatus may be divided broadly into two classes; the first in which the microscope is illuminated with monochromatic light[7,21] and the second in which all the radiation from the ultraviolet source passes through the achromatic microscope and is analysed by a spectrograph[14,22,23]. The first method, although applicable to microspectrographic studies on oriented structures, is mainly applied in the biological field where time of irradiation of the specimen must be kept to a minimum; while the second method is primarily suited to the study of non-living material. For the second method it is essential that the microscope be perfectly achromatic, and for this reason it has only been used with the reflecting microscope[24–29] which has the special characteristic of complete achromacy. Apart from the work of SIGNER, CASPERSSON and HAMMARSTEN[10] and the recent work of RUCH and THORELL[30] on oriented nucleic acid, all microspectrography in the field of structural chemistry has been carried out with the reflecting microscope and the second class of apparatus. In this method all the radiation, consisting in a line spectrum from the ultraviolet source (*viz.* mercury arc) is passed through the

specimen and the achromatic microscope throws a 'white light' image of the specimen on the spectrograph slit (the slit is adjusted to such a width that the images at various wavelengths do not overlap) and a series of images of the object are obtained at various wavelengths in the focal plane of the spectrograph as in the spectroheliograph. The method may be extended to use an ultraviolet continuum (*e.g.* the continuous spectrum of the hydrogen or xenon arc) and a narrow slit. The data may be recorded photographically and photoelectrically, but until now only the photographic method of recording has been used, the density measurements being obtained by microphotometry using either a rotating logarithmic or stepped sector to calibrate the photographic material. It is often stated that the accuracy of the photographic method of recording is only about 5 per cent, but this error appears to be of a random nature and may be considerably reduced by taking the mean of several measurements at different exposure times. We have found that with care the agreement between different measurements on the same specimen is about 1 per cent.

For structure analysis, using ultraviolet absorption spectroscopy, some form of polariser must be used and there are several suitable polarisers and double image prisms readily available. The polariser may be inserted either before or after the microscope and is probably placed most conveniently in the spectrograph. If inserted before the microscope, a polariser which does not deviate the ordinary ray is convenient and, by a suitable choice of angle of prism, the extraordinary ray may be deviated to such a degree that this ray is cut off by the condenser stop of the microscope. PERUTZ *et al.*[31] have used a Glan-Thompson prism as a polariser, whilst we[32] have found that a Rochon type calcite double image prism is suitable. However, in using calcite it must be remembered that the transmission falls off rapidly below λ2600Å; thus a 1 cm slab at λ2500Å absorbs approximately 50 per cent, while at λ2300Å the absorption is 95 per cent of the incident radiation. The most elegant method of obtaining dichroic data is undoubtedly by use of a double image polariser in the spectrograph. SCHEIBE *et al.*[13] have used a calcite plate, but we have found that a Wollaston double image quartz prism in which the two prisms are optically contacted is the most suitable, as this prism will transmit down to λ1750Å and the deviation of the rays is symmetrical. Unpolarised radiation is passed through the microscope and the double image prism is placed in front of the collimater lens of the spectrograph. The record consists of two series of images at different wavelengths polarised at right-angles to one another. With this system it is essential that there be no crystalline quartz between the object and the double image prism as the large optical rotation of quartz can cause grave errors. (Rotatory power of quartz at λ2500Å is 143° mm^{-1} and at λ3180Å it is only 84° mm^{-1}). There has been some attempt to use polaroid[33] as an ultraviolet polariser, but this work has

not been very successful, and in view of the advantage of the simultaneous recording of dichroic data by the double image method, it is felt that this method is the most suitable for the measurement of ultraviolet dichroism. Where a large field is required a pile of fused quartz coverslips mounted at approximately the Brewster angle makes a convenient polariser.

ACCURACY OF MICROSPECTROGRAPHIC MEASUREMENTS

A considerable amount of discussion has taken place on the accuracy of microspectrographic methods and apparatus, and there appears to be considerable difference of opinion on the requirements of the apparatus[34,35]. Clearly for accurate microspectrography it is essential that the aberrations of the microscope should be reduced to a minimum. However, as pointed out by WILKINS[36], in microspectrography, provided the objective has been adjusted so that no coma or astigmatism is apparent in the centre of the field, accurate measurements may be made provided spherical aberration is eliminated. Thus for microspectrography the object can always be placed at the centre of the field, and off-axis aberrations are in general of no consequence provided the system is spherically corrected (*cf.* CASPERSSON[34]). Using simple reflecting objectives constructed with spherical mirrors there is always some coma, except in the case of the monocentric pair[26], and with increasing aperture the field size diminishes rapidly; but it is possible to construct microscopes of about 0·6 Numerical Aperture whose field size is quite large enough for accurate microspectrography. Thus with simple spherical surfaces a reflecting objective of Numerical Aperture 0·6, and 35 per cent central obstruction of aperture, may be used with complete confidence in the accuracy of the results over a field of about 40 μ. (Error in wavefront less than $\lambda/8$ at λ2650Å[29]).

It might be expected that using a well-corrected objective the optical density of an opaque strip or disc measured by a microspectrographic method should approach infinity; in fact, when the experiment is carried out using every precaution to eliminate scattered light the optical densities measured[15] are approximately 1·5, which represents an apparent transmission of about 3 per cent in an object about 5λ wide. LORD RAYLEIGH[37] has shown that on diffraction theory the intensity of light outside the fourth dark ring is 4·7 per cent, and it can be shown[38] that for an opaque strip of semi-infinite width the intensity outside the fourth diffraction band is $\sim$ 3 per cent. The RAYLEIGH theory is based on Airy disc diffraction, and assumes incoherence, each point in the object behaving as a self-luminous point. In fact, however, in the microscope the effect of condenser aperture is to produce some degree of coherence and the above value probably represents the upper limit to the intensity of light scattered into a semi-

infinite sheet. A lower limit is obtained if coherence is assumed (*i.e.* parallel illumination); in this case the light diffracted outside the fourth dark band of a semi-infinite strip is ~ 0·3 per cent[39]. The values referred to above were calculated for the non-obstructed case; however, with reflecting objectives the central cone of light is always obstructed with resulting alteration in the diffraction pattern. The principal effect of this redistribution of energy is a diminution of the intensity of the central disc and an increase in the intensity of the first few diffraction rings. The effects of central obstruction become negligible beyond the 4th or 5th diffraction ring. Thus, for objects greater than 3 or 4 microns diameter the above figures should represent approximately the intensity of light diffracted into the object either in the non-obstructed or obstructed case. From these considerations it is obvious that microspectrographic measurements on objects of high optical density may be very inaccurate, while the effect on low density objects is negligible. Presumably the experimental values should lie between the theoretical values for the coherent and incoherent case, but until such time as a complete theory of microscopic image formation is obtained, measurements on objects of optical density greater than unity must be considered unreliable.

BLOUT *et al.*[40] have studied in detail the effects of condenser aperture on the optical thickness of specimens and have shown that the effects are only appreciable with high condenser apertures on objects of high density. Using condenser apertures of 0·3 to 0·4 N.A., the errors introduced by this effect can be neglected. A further source of error which may be appreciable with small objects is the 'phase contrast' effect due to the incorrect focus of the microscope. This effect has been considered by WILKINS[36], and may be avoided by the use of a perfectly achromatic microscope. It must be remembered that using a reflecting microscope the perfect achromacy of the instrument is only maintained when the object is mounted without a coverslip, and, as coverslips are generally used, an achromatising lens[25,29] must be inserted in the microscope to compensate for the chromatic aberration introduced by the coverslip.

EXPERIMENTAL

(1) *Preparation of specimens*

In general, substances which crystallise from solution are allowed to crystallise between coverslip and slide, and the thickness of the crystals can be controlled fairly well by adjusting the pressure on the coverslip. After some days, when the specimen has dried out, a normal paraffin (*viz.* n-hexadecane) with a refractive index approximating to that of the specimen is allowed to run in between the coverslip and slide to minimise the effects of reflection and refraction. Under these conditions of crystal

growth it is often possible to grow crystals on faces which are not the normal habit; thus with 4-6 dimethyl 2-hydroxypyrimidine-2H_2O crystals were obtained in which the pyrimidine planes were parallel to the surface of the slide and not perpendicular as is normally the case[14]. Some crystalline materials do not crystallise readily from solution and must be prepared under conditions of temperature control and *p*H which are almost impossible to simulate between a coverslip and slide, and occasionally crystalline materials must be maintained in suspension in the mother liquor. In such cases it is found preferable to mount a small drop of the crystalline suspension and seal the coverslip to the slide with a suitable wax or grease. It has been found that the fluorinated hydrocarbon, perfluorolube of *Dupont Limited*, is very suitable for this purpose and specimens mounted using this material do not dry out even after many weeks; using paraffin the suspension dried off after some days.

Oriented specimens of macromolecules, such as tobacco mosaic virus and desoxyribose nucleic acid, may be prepared by shearing of the specimen between coverslip and slide[31,32]. Using optically worked surfaces it is possible to obtain, with a little practice, very well-oriented sheets of uniform thickness several millimetres square and a few tenths of a micron thick. With microspectrographic methods it is always advisable to make measurements on regions surrounded by clear field, and the easiest way to prepare such specimens is to allow a well-oriented film, obtained by repeated shearing, to dry in air, and this dry film is then scratched with a sharp scalpel leaving narrow islands of the oriented material separated by clear regions. A coverslip is placed on the specimen and immersion oil (n-hexadecane) is allowed to run in to reduce the effects of reflection and refraction. Data may sometimes be obtained on the fibres drawn from gels of macromolecules or fibrous proteins. In general, measurements on such fibres are difficult as they are usually circular in section and, if the absorption coefficient is high, thin enough specimens are often too narrow for accurate microspectrophotometry. With such fibres immersion in a liquid of matching refractive index is essential if any reliance is to be placed on the data obtained.

(*ii*) *Dichroism of planar ultraviolet absorbing molecules*

Fundamental measurements to ascertain the nature of the dichroism of planar absorbing groups are very scarce, and it is only in recent years that some direct evidence on the dichroism of the benzene and pyrimidine ring has been obtained. Hexamethyl benzene crystallises with the planar molecules parallel to one another[41], and SCHEIBE *et al.*[13] have crystallised fairly large sheets of this material between quartz slides. In these preparations the plane of the benzene ring lies roughly normal to the quartz slide, and these authors have shown that the substance exhibits a large

dichroic ratio ($\sim$ 10:1 at λ2700Å) and that the radiation polarised with the electric vector parallel to the plane of the ring is preferentially absorbed (Figure 1). This is the first direct evidence of the ultraviolet dichroism of a planar molecule in the literature, although some circumstantial evidence from fluorescence spectra is reported. In recent years the structures of

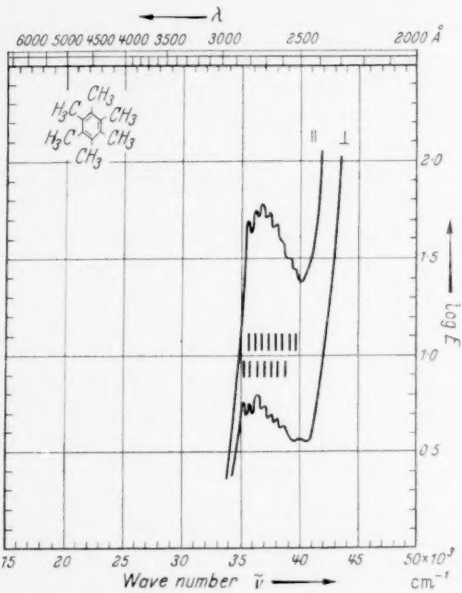

Figure 1. Ultraviolet dichroism of Hexamethylbenzene. ∥ Electric vector parallel, ⊥ electric vector perpendicular to the plane of the ring.
(SCHEIBE, HARTWIG and MÜLLER, *Z. Electrochemie* 49 (1943) 374)

several substituted pyrimidines have been obtained[42,43] by X-ray analysis, and the 4,6, dimethyl 2-hydroxypyrimidine-2H_2O, which crystallises with the molecules parallel to one another and pointing in the same direction[43], provides, as suggested by COULSON[44], an ideal material on which to study the ultraviolet absorption of the pyrimidine ring. Crystals of the compound are obtained from a 50 per cent aqueous alcohol solution, and under favourable conditions may be made to grow between coverslip and slide with the pyrimidine rings either parallel or perpendicular to the plane of the slide. It has thus been possible for the first time to obtain the three ultraviolet absorption spectra of a crystal, and although the effect is small, dichroism in the plane of the ring does occur. With this compound the whole absorption band is dichroic, and the dichroic ratio at λ3000Å may be as large as 100:1. Recently, LYONS[16] has measured the absorption spectra of some other compounds of the pyrimidine group using a technique similar to that described by SCHEIBE *et al.*[13] and his results are in general agreement with the others. These results show that in the case of the benzene and pyrimidine planar ring molecules the dichroism is

large throughout the whole absorption band and the dichroic ratio is very large (10 — 100 : 1). Until further evidence is obtained on other planar absorbing molecules or groups, it is clear that care must be taken in the interpretation of the dichroism of fine structure bands such as the characteristic fine structure absorption band in tryptophane at λ2890Å[32,46]. However, various workers have used the dichroism of this narrow absorption band to interpret the orientation of the tryptophane in tobacco mosaic virus[47] and haemoglobin[31], and until further work has been carried out on the absorption spectrum of the indole ring such interpretation may be incorrect. It is probable, in fact, that this narrow absorption band in the tryptophane spectrum represents an absorption perpendicular to the plane of the indole ring, and KASHA[48] suggests, on theoretical considerations, that this interpretation is possible.

A number of measurements[15] have been made on crystalline tryptophane and phenyl alanine in order to obtain some data on the dichroism of such absorbing systems, but the compounds do not crystallise in such a manner that they exhibit a large dichroism, and it is therefore probable that the absorbing groups are not oriented parallel to one another. BARER *et al.*[23] report some preliminary measurements on 1,2,5,6-dibenzanthracene but no details are given.

Microspectrographic observations have been made[32] on crystals of l-tyrosine crystallised between a coverslip and slide from a solution in warm distilled water. Crystallisation must occur slowly, and the necessary conditions are readily obtained by crystallisation in a vacuum flask. The crystals are flat needles which are positively birefringent with respect to the length of needle when viewed on the flat face, and negatively birefringent when viewed on edge. The dichroic measurements were made on the flat positively birefringent crystals and it was found that the whole absorption band from λ2500Å to λ3000Å is positively dichroic, but the dichroic ratio varies along the curve, being about 6 : 1 at λ2890Å. This large dichroism suggests that the benzene rings in the tyrosine crystal lie with their planes almost parallel to one another in the plane containing the refractive indices γ and β. Refractive index calculations[15] by the method of BUNN[49] confirm this suggested orientation of the benzene ring in l-tyrosine.

(*iii*) *Ultraviolet dichroism of non-crystalline biological macromolecules*

Tobacco mosaic virus (*T.M.V.*)—Chemical analyses[50] of the purified virus-associated nucleoprotein extracted from the cells of tobacco plants infected with tobacco mosaic virus indicate that the ultraviolet absorbing groups present are phenylalanine, tryptophane and tyrosine, and the ultraviolet absorption of the solution of the nucleoprotein agrees fairly well with the summation of the absorptions due to the constituent groups[47].

T.M.V. gel, in which the particles have aggregated end to end, is readily oriented into thin films by repeated shearing between coverslip and slide, and the solution of the nucleoprotein exhibits streaming birefringence[51]. The birefringence of oriented films is probably almost totally 'form birefringence' and, on thorough drying, the sheets are almost non-bire-

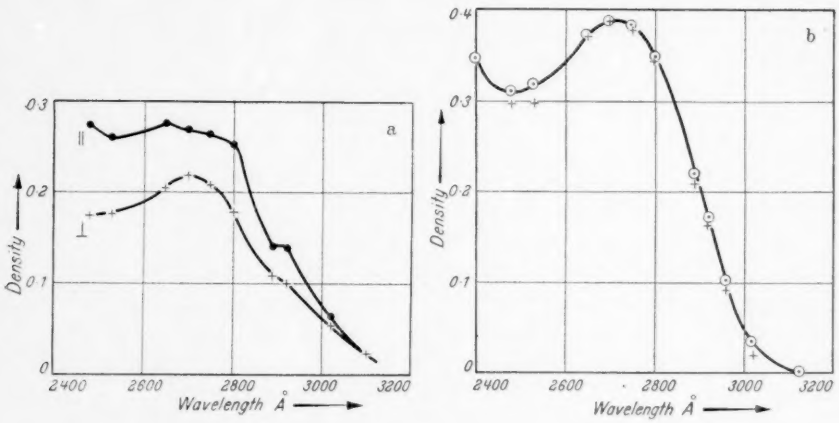

Figure 2. Ultraviolet absorption curves of tobacco mosaic virus: (*a*) Oriented film of purified T.M.V. gel. ∥ Electric vector parallel, ⊥ electric vector perpendicular to direction of shear (*b*) Hexagonal crystalline inclusions in leaf hair of T.M.V. infected tobacco plants. (SEEDS and WILKINS, 1950)

fringent[46]. Measurements of the ultraviolet dichroism on streaming solutions of the purified virus-associated nucleoprotein were made by BUTENANDT *et al.*[47], and it has been shown subsequently[46,31,32], by measurements both on streaming solutions and on dried oriented films, that though the general form of the absorption curves obtained by BUTENANDT *et al.*[47] are correct, the sign of the dichroism is wrong; *i.e.* the curves published by these authors must be interchanged with respect to the direction of the electric vector. There is a small dichroism of the whole absorption band, and the curve for the electric vector parallel to the length of the virus particle rods also shows the 'tryptophane notch', the characteristic fine structure absorption band of tryptophane at λ2900Å. BUTENANDT *et al.*[47] interpret these curves in terms of the orientation of the tryptophane and nucleic acid in the nucleoprotein, but as stated earlier, the interpretation of the dichroism of fine structure absorption bands is at present unjustified, and furthermore the dichroism at the maximum of the T.M.V. absorption curve is of the same order of magnitude and of the same sign as would be expected from form dichroism[32,46,52]. PERUTZ

et al.[31] have also pointed out that in tobacco mosaic virus the position of the 'tryptophane notch' is at λ2910Å as in most proteins, and not at λ2880Å as given by BUTENANDT *et al.* This latter position corresponds to the position of the band in free tryptophane. The ultraviolet absorption spectrum (ordinary ray) of the hexagonal crystals present in the leaf hairs and other cells of plants infected with T.M.V. has been measured[46,32], and this spectrum agrees well with that obtained on pure oriented virus-associated protein gel when the electric vector of the radiation is perpendicular to the rod-length of the virus particle (Figure 2). Thus, the rods of the nucleoprotein must lie normal to the hexagonal face of the crystal, confirming the results of WILKINS *et al.*[46] using polarised visible light; furthermore, this is the first direct evidence that the crystals are in fact the virus-associated nucleoprotein, although there was much circumstantial evidence to support this view[53].

Nucleic acid (DNA)—The sodium salt of thymus nucleic acid has for many years been known to be negatively birefringent, and this fact, together with the negative ultraviolet dichroism of films and streaming solutions[9,10,32,54], has been interpreted as indicating a long molecule with purine and pyrimidine bases lying roughly normal to its length. X-ray studies[55] have suggested that the molecule consists of a pile of flat nucleotides spaced some 3.4Å apart. Essentially this picture has not altered, although the suggestion by FURBERG[56] that the sugar rings are roughly planar and normal to the plane of the purine and pyrimidine is supported by his detailed x-ray analysis of a nucleoside. The sodium salt of highly polymerised thymus nucleic acid[57], when moistened with a small amount of water, swells greatly and forms a very viscous transparent mass showing between crossed polarisers a number of birefringent regions. This mass may be oriented readily either between coverslip and slide by repeated shearing, or by drawing into fine uniform fibres[54] some microns in diameter by slowly withdrawing a glass point from the mass of wet gel. This property of fibre formation appears to depend not only on the humidity, but varies from specimen to specimen. These fibres of nucleic acid are negatively birefringent and well oriented, but they are unsuitable for ultraviolet absorption measurements as the fibres are circular in section, and thin enough fibres (0·4 μ) are too narrow for accurate measurements. However, useful data may be obtained at the long wavelength tail of the absorption curve at λ2890Å or λ2960Å. These fibres will stretch to approximately double their length with permanent set, the birefringence of the resulting fibre being slightly positive. The positively birefringent fibres are readily turned back into negative fibres in the presence of water vapour[32,54]. The ultraviolet dichroism of both types of fibre is negative; *i.e.* the maximum absorption occurs for the electric vector perpendicular to the fibre axis, but in the case of the positively

birefringent fibre the dichroic ratio is much smaller[32]. Thus at λ2890Å, the air-dried negative fibres have dichroic ratio of about 1 : 1·75 and a birefringence of −0·08 in the visible; while for the positive fibres the corresponding figures are 1 : 1·05 — 1·1 and +0·02. The transformation from positive to negative fibres in the presence of water vapour has been carried out on the microscope stage, and in all cases measured, the dichroic ratio altered from 1 : 1·05 — 1·1 to 1 : 1·5 — 1·7 during the transformation. Microspectrographic absorption curves for well-oriented films of the sodium salt of highly polymerised nucleic acid have been obtained for light polarised perpendicular and parallel to the direction of shear[32,58]. These results confirm the results of CASPERSSON[11], and the maximum dichroic ratio observed at λ2600Å is about 1 : 1·7 at room humidity (~ 60 per cent). With slightly less polymerised material the dichroic ratio is slightly larger, ~ 1 : 2. The absorption curves show that the dichroic ratio is approximately constant throughout the whole of the absorption band. RUCH and THORELL[30] have made measurements on nucleic acid films and have found large dichroic ratios (~ 1:3·2) for freshly prepared specimens. We[58] have made fairly detailed observations on the effects of humidity on the dichroism of oriented nucleic acid. An oriented film of sodium salt of DNA was mounted on a coverslip over a small cell containing saturated solutions of various salts giving constant humidity conditions, and measurements were made on the same specimens under a humidity range of 40 per cent to 98 per cent. It was found that oriented nucleic acid in equilibrium with an atmosphere of less than 80-90 per cent humidity is quite stable to irradiation, while at humidities greater than 90 per cent the orientation of the specimen rapidly decreases, the birefringence and ultraviolet dichroism falling to zero. This effect occurs over a considerably larger area than the actual area irradiated. The dichroism of the nucleic acid increases with increasing humidity and values of the dichroic ratio of 1:4 are not uncommon, and on one specimen a dichroic ratio of 1:4·7 has been observed (Figure 3). In the figure the two absorption curves of the same region of oriented nucleic acid are given under two different conditions of humidity. If it is assumed that in these oriented films of nucleic acid the molecules do not possess rotational symmetry about their axes, and that the nitrogen bases are so arranged that the specimen presents a 'fishbone' appearance, the variation in dichroic ratio from 1:1·9 to 1:4·7 as the humidity increases from 70 per cent to 90 per cent indicates that on the average, with increasing humidity, the nitrogen bases have oriented themselves more nearly normal to the molecular axis; the actual mean change in angle is from 36° to 26° to the normal. (If the molecules possess rotational symmetry the variation in angle to the normal is from 40° to 27°). Thus the ultraviolet dichroism indicates that in the nucleic acid molecule the purine and pyrimidine bases are so arranged that on the average they

lie at a small angle to the normal to the molecular axis, and that this angle decreases with increasing humidity.

Calf thymus nucleoprotein—The nucleoprotein of calf thymus may be oriented into films and fibres in a manner similar to the nucleic acid,

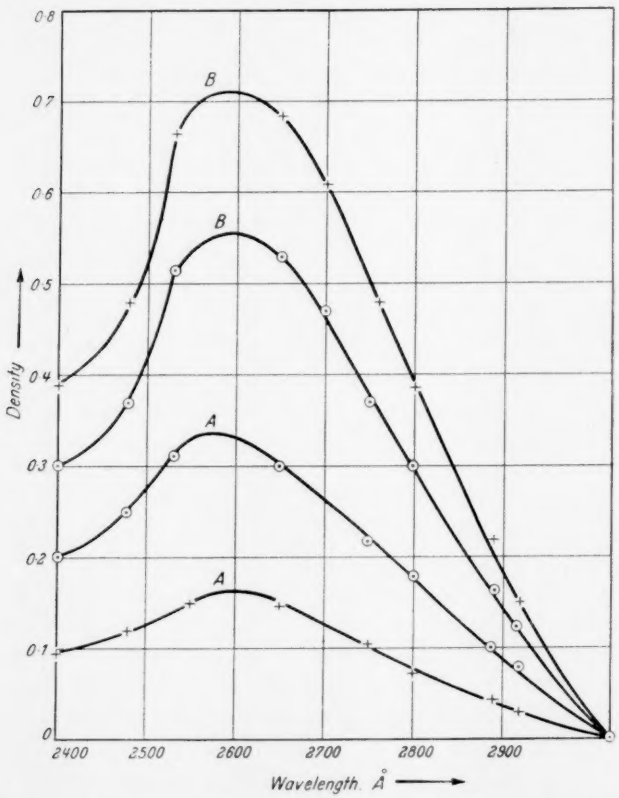

Figure 3. Ultraviolet absorption curves of oriented film of the sodium salt of thymus nucleic acid under different conditions of humidity: (A) Electric vector parallel, and (B) Electric vector perpendicular to direction of shear. (+) at 90 per cent humidity and (⊙) at 60 per cent humidity. (SEEDS and WILKINS, 1951)

although this orientation does not occur as readily as with purified nucleic acid, and the films and fibres tend to be uneven, thus making accurate absorption measurements difficult[32]. The nucleoprotein is negatively birefringent and dichroic. Dichroic ratios of 1:1·5 — 1·7 have been obtained on air-dried specimens of birefringence —0·014, and as for nucleic acid, the maximum absorption occurs when the light is polarised with the electric vector perpendicular to the direction of shear. These observations suggest

that the ultraviolet absorbing groups in the nucleoprotein are oriented in a manner similar to the arrangement in the nucleic acid itself.

Non-crystalline proteins of the Keratin type—A number of proteins of the α and β keratin types have been examined in ultraviolet radiation, but most of these do not exhibit any pleochroic effects. PERUTZ *et al.*[31] have observed that porcupine quill tip, frog sartorious muscle, and stretched films of tropomyosin and fibrinogen do not exhibit any measurable dichroism, and it has also been shown[58] that wool, feather keratin and elephant tail hairs have almost zero dichroism. Measurements on these types of material are always difficult as specimens must be prepared by sectioning according to normal biological techniques and it is possible that some disorientation occurs and the preparations are never uniform.

During recent years SCHAUENSTEIN and his colleagues at Graz have made a series of observations on the ultraviolet absorption of fibrous proteins using the method of SCHEIBE *et al.*[13], and they claim to have observed an absorption between λ2300Å and λ5000Å in collagen, silk fibroin, and actomyosin which they attribute to the absorption of the peptide grid[59–61]. These workers claim that the peptide absorption curve has a flat contour with smeared out maxima at λ2500Å and λ4000Å, and that the intensity of this band depends on the mechanical deformation of the specimen. The absorption of this band increases with increasing deformation. SCHAUENSTEIN points out the connection between this 'additional absorption' and the hydrogen bonding and suggests that the absorption can be ascribed to the enolisation of the peptide group; *i.e.* the absorption is due to the formation of the $-C = N-$ group, and this

$$\begin{array}{c} \\ | \\ \text{OH} \end{array}$$

absorption is referred to as 'peptenol absorption'. The experimental results reported by KRATKY[61] on silk fibroin are difficult to explain if the laws of electromagnetism are to be obeyed[62]. Thus, he claims that the stretched silk, an anisotropic material whose properties correspond to a uniaxial crystal, is characterised by three absorption curves, one for the vector parallel to the axis, one for the vector perpendicular to the axis, and a third for the vector at 43° to the axis. Furthermore, this third absorption is greater than either of the other two. It is probable that the phenomena described are not due to a real dichroism and may be similar to some observations which WILKINS and SEEDS[58] have made with polarised visible light on stretched silk fibroin sheets. Stretched sheets of fibroin between crossed polarisers give, in certain regions, what appeared to be anomalous colours, which effects are apparently due to reflection from the surface of the film in those parts where it was not lying flat on the slide. On immersion in paraffin, reflection is reduced and these anomalous colours, which give an apparent dichroism, disappear. Measurements on

collagen by FIXL *et al.*[60] are also claimed to indicate this additional absorption of the peptide at an angle of about 50° to the axis of the collagen fibre.

Measurements of the ultraviolet absorption of such materials as collagen are difficult as, owing to the nature of the specimen, much of the light is scattered, and as many of these materials contain few ultraviolet absorbing groups the specimens are of necessity rather thick. Until further detailed work has been carried out on these materials the phenomena described should be considered very critically. There is some evidence from infra-red studies that this so-called enolisation of the peptide grid does not occur. ELLIOTT[63] has shown, using polarised infra-red, that the infra-red spectrum of silk fibroin does not contain any bands which might be attributed to the bands of the so-called peptenol group, and it is likely that if this group were present there should be some positive indication in the infra-red absorption.

(*iv*) *Crystals of biological origin*

Haemoglobin—The visible and ultraviolet absorption spectrum of crystals of horse methaemoglobin have been obtained by PERUTZ *et al.*[31]. The crystals examined were about 3 μ thick and are plate-like, growing on a face containing the a and b axis of the crystal. These crystals exhibit a strong visible dichroism[64]. The absorption curves (Figure 4) show a marked dichroism of the Soret band at λ4000Å, the greatest absorption being for the radiation polarised with the electric vector parallel to the axis of the crystal. The two absorption curves approach one another at λ3100Å and the dichroism rises to another maximum at λ2900Å, remaining fairly large down to λ2660Å, the shortest wavelength at which measurements were made. The sign of the dichroism of the Soret band is the same as that of the absorption bands of haemoglobin in the visible, and this absorption appears to be greatest when the light is polarised with the electric vector parallel to the plane of the haem group. The haem group shows no absorption maxima on the short wavelength side of λ3100Å so it may be assumed that the dichroic absorption below λ3000Å is due to the aromatic amino acids. Considering the relative extinctions of the three ultraviolet absorbing amino acids present in haemoglobin (*i.e.* tryptophane, tyrosine and phenylalanine) there is little doubt that the fairly large dichroic ratio ($\sim$ 1:3) at 2900Å is due to tryptophane but it is difficult to decide whether the dichroism of the remainder of the absorption band is due to tryptophane alone, or to a preferred orientation of the other aromatic side-chains. PERUTZ[65] has shown that the polypeptide chains lie roughly parallel to the 'a' axis of the crystal. Thus, if one assumes that the dichroism below λ3100Å is due to the orientation of the aromatic rings, these rings would lie at a small angle to the normal to the chain

axis, or roughly parallel to the haem groups. However, as PERUTZ[31] agrees, the interpretation of the dichroism of the tryptophane band at λ2900Å is not certain, and any interpretation of the orientation of tryptophane from this band must not be considered as conclusive.

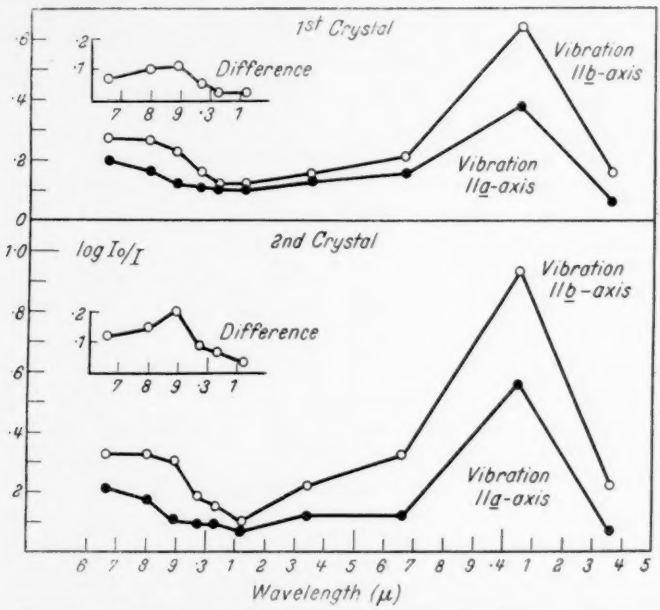

Figure 4. Absorption curves of two single crystals of horse methaemoglobin. Electric vectors of polarised light parallel to the a- and b-axis of the crystal.
(PERUTZ, JOPE and BARER, *Discuss. Faraday Soc.* No. 9 (1950)

Ribonuclease—Crystalline ribonuclease[66] is a soluble protein of the albumin type obtained from beef pancreas and is crystallised from aqueous alcohol solution into fine needles or rectangular plates[67]. This protein contains tyrosine but no tryptophane and its ultraviolet absorption is due to the tyrosine side groups. A specimen of ribonuclease prepared by KUNITZ contained a variety of crystal forms, the majority of the crystals growing on the 001 or 100 face[68,69]. Both these crystals exhibit straight extinction and are positively birefringent with respect to the direction of γ (parallel to the crystallographic 'b' axis[58,69,70]). The ultraviolet absorption of both types of crystal shows a weak dichroism[58], the maximum absorption occurring when the radiation is polarised with the electric vector perpendicular to the 'b' axis. Thus, the ultraviolet groups must lie roughly in the crystallographic plane 'b' containing the refractive indices α and β. At the maximum of the tyrosine absorption the dichroic ratios observed in both cases are approximately 1:1·5 indicating that the planes

of the tyrosine rings must lie tilted on the average at an angle of 40° to the plane 'b'. It has been noted[58] that there is a tendency for the crystals to break down and become non-dichroic and non-birefringent under irradiation. The disoriented area corresponds to the actual area irradiated (*cf.* nucleic acid at 90 per cent humidity); thus if a narrow band across a crystal is irradiated, after some time the crystal, if viewed on the polarising microscope, is crossed by a non-birefringent band.

Vitamin B_{12}—A series of measurements have been made on the ultraviolet absorption of the anti-pernicious anaemia factor of B_{21}[58] to try to decide between alternate structures suggested by x-ray diffraction[71,72]. This compound contains a group similar in structure to a porphyrin but containing a central cobalt atom, a benziminazole group, a sugar, and some smaller organic groups. The ultraviolet absorption of the compound in the region λ2500Å — λ3000Å is in part due to the pseudo-porphyrin and in part to the benziminazole group, the contributions from the two groups being roughly equal[73]. The crystals are readily grown from aqueous solution between slide and coverslip, are red, and in certain forms exhibit a very marked visible dichroism due to the orientation of the porphyrin type group[71,72]. It was hoped that ultraviolet dichroic measurements would decide whether the plane of the benziminazole group in the crystal is oriented parallel or perpendicular to the plane of the pseudo-porphyrin. The absorption spectrum of the benziminazole fragment of the compound[73] is complicated and has three maxima at λ2880Å, λ2800Å and λ2740Å; furthermore, as the group contains nitrogen atoms it is probable that some of this spectrum is due to vibrations perpendicular to the plane of the group, assuming that in fact this group is planar. All the crystals examined[58] exhibited some dichroism throughout the whole ultraviolet region and the dichroism is of the same sign as the visible or near ultraviolet dichroism. But until detailed absorption data, both on the pseudo-porphyrin and on the benziminazole group, have been obtained the interpretation of the results may only be tentative. From the point of view of microspectrography, this work of a somewhat negative nature is of interest as an example of the limitation of the method when the absorption spectra of various groups in a large molecule overlap, and the molecular spectra are complex.

Other crystalline biological material:

Ultraviolet absorption curves for crystalline lysozyme[74], a low molecular weight protein containing tryptophane[75], have been obtained[15] and a small dichroic ratio ($\sim$ 1:1·4) has been observed on the needle-shaped crystals obtained from a 5 per cent NaCl solution at *p*H 9·5. These crystals are positively birefringent and further measurements will be made when more detailed x-ray analyses are available.

Crystalline gramicidin (WALLERSTEIN) has been examined in the ultraviolet, and crystals obtained from 95 per cent alcohol show a marked

dichroism[15]. This particular gramicidin is a mixture of A and B, but the x-ray diffraction of these crystals does not differ from the pattern for pure gramicidin A[72,76]. The ultraviolet absorption of gramicidin A is almost totally due to the tryptophane content, and it may be possible from these measurements to obtain some evidence of the nature of the dichroism of the indole ring.

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3

THE INFRA-RED SPECTRA OF BIOLOGICALLY IMPORTANT MOLECULES

R. D. B. Fraser

ANY account of the infra-red spectra of biologically important molecules necessarily relates largely to polymers. In an article of this length it would be out of place to discuss the spectra of the lower molecular weight compounds; they do not, in general, present features of special interest which distinguish them from other organic compounds. The standard methods of infra-red structure analysis[1,2,3] and identification or estimation employed with them need not be reiterated here. The special problems and difficulties that have been encountered in the study of the infra-red spectra of biologically important macromolecules are best appreciated by considering the manner in which the absorption of infra-red radiation takes place.

THE NATURE OF INFRA-RED SPECTRA

In studying the vibrational motions of an isolated molecule, it is customary to treat the molecule as a system of point masses maintained in an equilibrium configuration by elastic forces between the masses. Any displacement from this equilibrium configuration will result in a vibrational motion which may be represented as a combination of a limited number of normal modes of vibration. A non-linear molecule containing n atoms possesses 3n-6 normal modes, some of which will be associated with a periodic variation of the electric dipole moment of the molecule. These are the modes which may be excited by the absorption of infra-red radiation of the appropriate frequency. The infra-red absorption spectrum of a molecule therefore comprises a number of fundamental vibrational frequencies together with certain overtones and combinations. These frequencies are usually expressed in wave numbers ($cm.^{-1}$) which are the actual vibrational frequencies divided by the velocity of light in vacuo.

For many simple molecules a complete interpretation of the absorption spectrum has been accomplished in terms of the normal modes of vibration[4]. In the case of a biological polymer, however, an analysis involving a knowledge of the locations of all the atoms and the nature of the forces maintaining them in their equilibrium configuration, is not possible.

However, it has been shown that although a fundamental frequency involves, to some extent, all the atoms in the polymer, certain vibrations are localised to a high degree of approximation in a particular bond or atomic grouping[5]. These characteristic vibrational frequencies associated with particular atomic groupings persist, with minor modifications, in any molecule containing that grouping. Characteristic frequencies are to be expected (*a*) for vibrations involving hydrogen atoms where adjacent atoms are much heavier, although the bond stretching and bending force constants may be similar, and (*b*) where the force constants between two atoms are very different from those between neighbouring atoms, even though the masses may be comparable. For example, the $>C = O$ grouping has a very characteristic stretching frequency, whilst this is not true for C—C or C—N in the grouping $-\overset{|}{\underset{|}{C}} - \overset{|}{\underset{|}{C}} - N<$.

The types of fundamental frequencies that are readily recognised in the spectrum of a macromolecule may be classified as follows:

1. *Vibrations involving hydrogen atoms*

(*a*) bond stretching	OH	3,000 — 3,700 cm.$^{-1}$
	NH	3,000 — 3,500 cm.$^{-1}$
	CH	2,800 — 3,100 cm.$^{-1}$
	SH	*ca.* 2,500 cm.$^{-1}$
(*b*) bond bending	OH	*ca.* 1,100 cm.$^{-1}$
	NH	1,500 — 1,600 cm.$^{-1}$
	CH	1,300 — 1,500 cm.$^{-1}$

2. *Vibrations involving multiple-bond stretching*

C = O	1,600 — 1,800 cm.$^{-1}$
C = N	*ca.* 1,650 cm.$^{-1}$
C = C	*ca.* 1,650 cm.$^{-1}$
P = O	1,250 — 1,300 cm.$^{-1}$

3. *Vibrational motions involving comparable motions of many atoms*

These are often referred to as skeletal frequencies and generally occur below 1,300 cm.$^{-1}$. There are certain skeletal frequencies, however, which persist from molecule to molecule; *e.g.* absorption bands near 1,600 cm.$^{-1}$ are found with compounds containing aromatic nuclei, and the $\geqslant C—O—C \leqslant$ grouping gives rise to an absorption band near 1,100 cm.$^{-1}$. With a few exceptions, however, the presence of an absorption band below 1,300 cm.$^{-1}$ can rarely be associated with the presence of a particular grouping of atoms.

4. *Vibrations localised in ionic groupings*

Where a macromolecule contains ionic groups such as PO_4^{---} and CO_3^{--}

these can generally be recognised as they give rise to broad absorption bands at well known frequencies.

PO_4^{---}	*ca.* 1,080 and 980 cm.$^{-1}$
CO_3^{--}	*ca.* 1,400 and 880 cm.$^{-1}$
SO_4^{--}	*ca.* 1,100 and 450 cm.$^{-1}$
NO_3^{-}	*ca.* 1,380 and 840 cm.$^{-1}$

Detailed summaries of these correlations have been given by COLTHUP[6] and others[7,8,9,10].

TOPICS OF SPECIAL INTEREST IN THE SPECTRA OF MACROMOLECULES

Hydrogen bonding

The characteristic frequency of the O—H stretching-vibration occurs near 3,650 cm.$^{-1}$; if the hydrogen atom is sufficiently close, however, to a carbonyl (C = O) group for the force field to be influenced by the oxygen atom, a weak association, or hydrogen bond is formed. The effect of this association is to reduce the stretching constant of the OH group, giving a broad absorption band at lower frequencies[11]. Hydrogen bonds in biological polymers have been detected spectroscopically for several combinations of groupings, including O—H....O, O—H....O=C, N—H....O=C, NH_2....O=C.

When hydrogen bonding is present the absorption band associated with the NH or OH stretching vibration is considerably broader than that of the corresponding 'free' vibration. The precise explanation of this is not known, but it seems probable that it is due, in part, to the influence of thermal agitation, varying molecular environment, and interactions with other vibrational modes. This results in a distribution of force constants with a statistical average corresponding to the observed absorption maximum. An alternative explanation has been put forward by CANNON[12] in terms of a double minimum potential energy curve of the proton between the bonded atoms.

The frequency shift caused by hydrogen bonding is considerable; *e.g.* in fibrous proteins the NH stretching frequency is about 100 cm.$^{-1}$ below the 'free' value of 3,400 cm.$^{-1}$ [13]. This shift is generally taken as a measure of the strength of the hydrogen bond. This is not strictly true, since the 'free' value for that particular compound can rarely be measured. Other factors can influence the frequency of this NH vibration; for example the corresponding frequency in collagen is 3,330 cm.$^{-1}$ [14] *i.e.* 70 cm.$^{-1}$ below 3,400 cm.$^{-1}$. Whilst the 30 cm.$^{-1}$ difference may be due to a weaker system of hydrogen bonding it could also arise from a difference in the electronic structure of the NH bond associated with a distortion from planarity of the amide group[15]. The restriction of resonance resulting

from such a distortion would certainly account for this. A second example is found in the absorption band near 3,100 cm.$^{-1}$ observed in the spectra of fibrous proteins, including collagen. This band is considerably weaker than the NH band near 3,300 cm.$^{-1}$, but is almost certainly an NH stretching mode of some kind[16]. Originally it was assigned to NH groups involved in a very much stronger system of hydrogen bonding[17]. Although this explanation is no longer tenable[18], a completely satisfactory interpretation has not yet been given. It has been suggested that the splitting arises from an interaction between identical N—H . . . . .O=C groupings[19] in the more crystalline regions of the protein fibre. This suggestion is supported by the observation that the lower frequency component, which is also observed in Nylon, is reduced in intensity as the temperature is increased[18].

It is clear that whilst important information can be gathered from the observation that a particular vibration is shifted in frequency due to hydrogen bonding, considerable caution must be exercised in relating the magnitude of the shift to the strength of the hydrogen bond.

The assignment of vibrations

The number and diversity of atomic groupings in a biological polymer leads to a multiplicity of both characteristic and skeletal frequencies. Whilst it is possible to assign all the absorption bands of many simple molecules to specific modes of vibration, it is rarely possible to assign, with any certainty, more than a few of the characteristic vibrational frequencies in the polymers. Apart from the extensive overlapping of absorptions, assignment is further complicated by the breadth of the absorption bands arising from hydrogen bonded groups, and the partial obscuration of two regions of great interest by any water that remains in the specimen. The OH stretching vibrations in water give rise to an extremely broad and intense band extending from 2,800 — 3,800 cm.$^{-1}$, with a maximum near 3,400 cm.$^{-1}$, and the OH bending vibrations a lesser band near 1,640 cm.$^{-1}$ [20]. Inhomogeneity in the specimen is also an important factor which may contribute to the general broadness of the absorption bands. The care necessary in the preparation of optically homogeneous specimens of uniform thickness is invariably amply rewarded.

Between 650 and 1,300 cm.$^{-1}$ the principal contributions to the absorption spectrum are from vibrations of the molecular framework; *i.e.* skeletal modes. These are so numerous that this is generally a region of more or less continuous absorption with an occasional maximum marking the position of a particularly intense absorption. Exact assignment of these maxima is possible only in special cases; *e.g.* the symmetric and antisymmetric vibrations of the phosphate groups in nucleic acid may be

recognised near 980 and 1,100 cm.$^{-1}$ respectively. DARMON[17] has succeeded in correlating certain absorption bands in silk fibroin with those in polyglycine and poly-L-alanine.

In the region 1,300 — 2,000 cm.$^{-1}$ a number of absorption bands can generally be assigned with a fair degree of certainty. Where the polymer contains carbonyl groups, one or more intense absorptions are observed between 1,550 and 2,000 cm.$^{-1}$ arising from the stretching vibrations of these groups. The exact frequencies of the absorption maxima are closely dependent upon the molecular environment; the C=O stretching frequencies in ketones, aldehydes, amides *etc.* occur within small frequency ranges that are well-known[6]. Deviations occur when the carbonyl group is conjugated or involved in hydrogen bonding. In both cases the shift is to lower frequencies. Once again the magnitude of the shift is often correlated with strength of the hydrogen bond but, as with the hydrogen stretching vibrations, the results must be regarded as semi-quantitative. Other double-bond stretching vibrations (C=C, C=N) are less readily assigned, partly because they are not end groups and partly because the effects of conjugation are more difficult to predict. In open chains both these vibrations occur near 1,650 cm.$^{-1}$, but are extremely variable in cyclic compounds. In both cases the effect of conjugation is to reduce the force constant between the atoms so that the requirement for a characteristic vibrational frequency mentioned previously is not completely satisfied, and the vibration ceases to be highly localised about the double bond.

Certain CH_2 and NH deformation modes may be assigned in this region. The CH_2 absorption occurs near 1,450 cm^{-1} and, as with the carbonyl group, the exact location can be correlated with other substituents around the carbon atom. CH_2 deformation frequencies are also affected by conjugation. In simple amines the in plane deformation frequency of the NH_2 group occurs in the range 1,590 — 1,650 cm.$^{-1}$, but very little is known with certainty about the frequencies in amides and other amines. The assignment of the band near 1,550 cm.$^{-1}$ observed in the spectra of monosubstituted amides is of particular importance with regard to the spectra of proteins. Although this is often assumed to be a characteristic vibration of the NH group[19], the partly double-bond character of the C—N link in the amide group[21] suggests that this may not be so. The comparable frequencies of the C = N stretching and NH deformation vibrations may in fact result in a complicated mode involving the whole amide group[22,69,70]. Recently, evidence has been obtained which supports the assignment to an NH deformation mode[8], but the question remains open. NH deformation frequencies are affected by hydrogen bonding, being shifted to higher frequencies, and are sensitive to the changes in the electronic structure of the bond.

Apart from overtone and combination bands which are usually weak, any band in the spectral interval 2,500 — 3,800 cm.$^{-1}$ may confidently be assigned to a hydrogen stretching vibration. The CH stretching vibrations are easily recognised, for they are found at well defined frequencies near 3,000 cm.$^{-1}$ depending upon the type of bonding between the parent carbon atom and its immediate neighbours[6]. Most biological polymers have a pair of bands between 2,800 and 3,000 cm.$^{-1}$ arising from alkane groups, with a further weak band between 3,000 and 3,100 cm.$^{-1}$ if the polymer contains alkene or aromatic groups. The 'free' OH and NH vibrations occur as sharp peaks near 3,680 and 3,400 cm.$^{-1}$ respectively, whilst the NH_2 groups gives a pair of sharp bands near 3,300 and 3,400 cm.$^{-1}$, corresponding to the symmetric and antisymmetric modes of vibration. All these frequencies are reduced by hydrogen bonding, so that the assignment of bands between 3,000 and 3,400 cm.$^{-1}$ presents great difficulty in cases where a polymer contains significant concentrations of more than one of these groups. The matter is further complicated by the increase in breadth of absorption bands accompanying hydrogen bonding, so that weak bands appear only as points of inflexion when they overlap stronger bands. Considerable assistance in the elucidation of overlapping absorptions in this region may be gained by thorough drying in vacuo, to reduce the broad absorption of water. Other possibilities consist in exchanging the labile protons with deuterium in heavy water solutions[20], which reduces hydrogen stretching frequencies in the ratio of about $1/\sqrt{2}$, and selective substitution of one particular group by chemical methods; *e.g.* acetylation or nitration[23].

INFRA-RED DICHROISM

It was mentioned previously that the normal modes of vibration of a molecule that may be excited by infra-red radiation are those accompanied by a periodic variation in the electric moment of the molecule. The oscillating moment has a unique direction with respect to the molecule and may be represented by a vector P. In a uniformly oriented structure where all these vectors P are parallel the extinction coefficient for that vibration will depend upon the angle between P and the incident electric vector E. The optical density will, in fact, be proportional to the squared scalar product of E and P. By the use of polarised radiation this infra-red dichroism may be detected and the sense and magnitude of the dichroic ratio related to the direction of P[24–29].

Instrumentation

Investigations of infra-red dichroism have been restricted in the past by the difficulty experienced in obtaining suitable polarisers. Polarised infra-red radiation may be obtained by reflection from selenium at the Brewster

angle[30], but the optical arrangements so far described are cumbrous and difficulty is experienced in rotating the plane of polarisation. More recently, compact transmission polarisers have been used. These comprise a pile of thin plates transparent within the spectral interval required, the number of plates depending upon the refractive index of the material and the degree of polarisation required[31].

VALLANCE-JONES[26] has described a polariser in which silver chloride sheets are used; an attempt is made to compensate for the varying angles of incidence in the convergent beam falling on the slit of the spectrometer. In the author's experience, this type of polariser is robust and easily constructed[32], but is not suitable for accurate work, owing to the image displacement introduced and the scatter from the non-optical surfaces of the silver chloride sheets. These have been overcome in the polariser described by ELLIOT *et al.*[33], in which extremely thin films of selenium are used. Considerable care, however, is necessary in the preparation and handling of the evaporated films.

Although dichroism measurements may be made with commercially available spectrometers, the specimen dimensions required (*ca.* 1 × 0·1 cm.) are large. Great difficulty is experienced in the preparation of uniformly oriented specimens of this size. Reflecting microscopes have been used in conjunction with infra-red spectrometers so that measurements of dichroism may be made on very much smaller areas. The simplest of these consists of a pair of off-axis parabaloids giving a linear magnification of 3 ×[34]. For higher magnifications (× 10 — 20) concentric mirror pairs have been used by BLOUT *et al.*[35] and the author[36]. A typical arrangement, now in use in this laboratory is depicted in Figure 1.

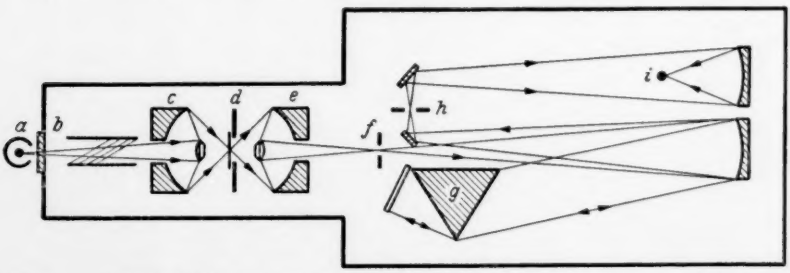


Figure 1. A typical infra-red microspectrometer used for measurements of infra-red dichroism in biological polymer specimens of microscopic dimensions. *a.* Nernst filament, *b.* selenium polariser, *c.* 0.8 N.A. reflecting condenser, *d.* object on stage, *e.* reflecting objective, *f.* entrance slit, *g.* NaCl or LiF prism, *h.* exit slit, *i.* thermocouple. The instrument is completely enclosed and dessicated to reduce absorption due to atmospheric water vapour.

The interpretation of dichroism

The results of dichroism measurements on oriented biological materials are generally expressed in terms of a dichroic ratio equal to the ratio of

the optical densities measured with the incident electric vector vibrating parallel and perpendicular to a certain reference direction in the specimen. In singly oriented polymers this direction is generally the axis in the case of a fibre, or the direction of shear or rolling in the case of a film. In crystalline specimens the optical density is determined for a number of crystal orientations, depending on the symmetry class to which the crystal belongs[29].

The relation between dichroic ratio and the direction of transition moment has been given for crystals[29], doubly oriented polymers[27], and singly oriented polymers[28]. In each case, however, the effects of convergence in the incident radiation were neglected, but the use of highly convergent radiation cones in a reflecting microscope introduces small but significant errors in the measured optical densities.

The first of these errors arises from the increased path length in the specimen of oblique rays. A treatment of this error has been given by BLOUT *et al.*[37], and a table of corrections is given in terms of the ratio of the numerical aperture of the incident radiation cones to the refractive index. The second error introduced by the oblique rays is a component of the incident energy in which the electric vector vibrates along the optic axis of the microscope. The magnitude of this component has been calculated and a corrected form of the relation between dichroic ratio and direction of transition moment given for the case of a singly oriented polymer[28]. As this is the case most commonly encountered the full expression will be given:

$$2\cot^2\beta = \frac{\log_{10}\left[T\pi + M\left(T\pi - T\sigma\right)\right]}{\log_{10} T\sigma}$$

where β = Inclination of vector P to axis
$T\pi$ = Ratio of transmitted to incident energy with electric vector parallel to axis
$T\sigma$ = Ratio of transmitted to incident energy with electric vector perpendicular to axis

$$\text{and } M = \frac{3}{2}\frac{(\cos\theta_1 + \cos\theta_2)}{(\cos^2\theta_1 + \cos^2\theta_2 + \cos\theta_1\cos\theta_2)} - 1$$

where θ_1 and θ_2 are the semi-angles including the radiation cone in the specimen.

RECENT ADVANCES

Prior to 1947 little attention was given to the spectra of biologically important molecules, and any discussion of recent advances in this field necessarily includes almost all the important contributions to date. A

detailed discussion of all these investigations is not possible in an article of this length, and only the main features will be mentioned. A fairly complete list of references is given, and where more detailed information is required reference should be made to the original papers.

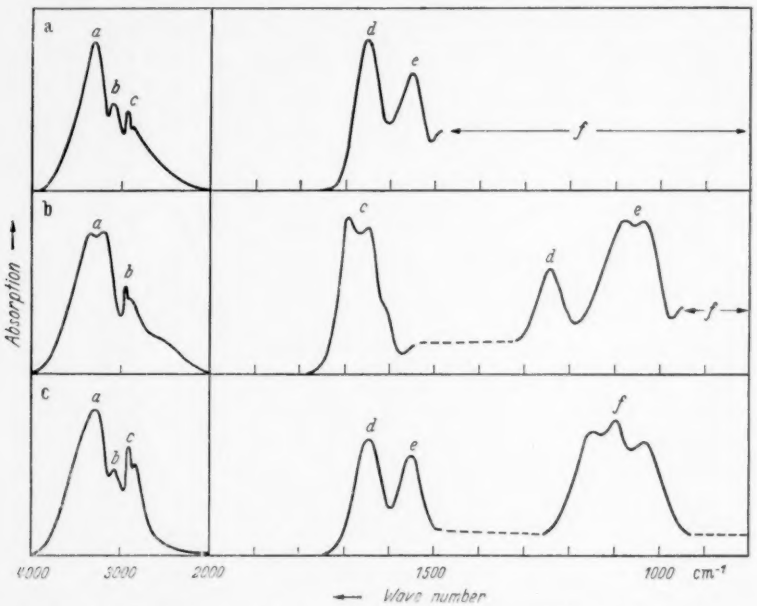


Figure 2. A diagram summarising the chief infra-red absorption bands observed in *a*. Proteins. *b*. Nucleic Acids. *c*. Polysaccharides.

Proteins

The most important absorptions observed in the spectra of both fibrous and globular proteins are depicted diagrammatically in Figure 2*a*. LENORMANT[38] noted that bands *d* and *e* appeared in the spectra of a wide variety of proteins; they were assigned by DARMON and SUTHERLAND[39] to the carbonyl stretching and NH bending vibrations of amide groups respectively. These authors also assigned bands *a* and *b* to hydrogen bonded NH stretching vibrations, and band *c* to CH stretching modes. The absorption spectrum between 800 and 1,500 cm.$^{-1}$ (*f*) is characteristic of the particular protein and attempts have been made[17, 40] to correlate it with the amino acid composition. A list of references in which spectra of proteins are reported is given in *Table I*.

The main NH stretching peak (a) is at 3,300 cm.$^{-1}$ and little variation of frequency occurs between different proteins or in the α — β transformation[54]. The only exception so far observed is in the case of the collagen-gelatine group of proteins[13,14]. The slightly higher frequency of

Table I

		References
Proteins	Actomyosin	45, 51
	Collagen	13, 49
	Chymotrypsin	66
	Cytochrome c	40
	Gelatine	13
	Glyoxalase	66
	Histone	48
	Insulin	46, 66
	Lysozyme	40
	Keratin	13, 42, 52
	Methaemoglobin	44, 66
	Myosin	42, 47
	Nucleohistone	37
	Pepsin	40
	Ribonuclease	48, 67
	Salmine	40
	Serum Albumin	40, 41
	Silk	13, 17, 43, 50
	Tropomyosin	17, 42
Nucleic Acids	Ribose Nucleic Acid	60, 61, 62, 63
	Deoxyribose Nucleic Acid	59, 60, 61, 62, 63
Polysaccharides	Alginic Acid	7
	Cellulose	7, 37, 64
	Chitin	64
	Hyaluronic Acid	68
	Lignin	65

3,330 cm.$^{-1}$ may be due to a different type of hydrogen bonding, or to a distortion from planarity of the amide group[15]. In thermally contracted collagen[15] and in gelatine films evaporated from hot solutions[55] a frequency shift to *ca.* 3,300 cm.$^{-1}$ is observed. It seems likely that this is due to a change in the configuration of the polypeptide chains, possibly to the α-form[15].

The weaker NH stretching peak (b) was originally assigned to strongly hydrogen bonded groups[17], and the frequency (*ca.* 3,070 cm.$^{-1}$) associated with a closed ring in which two amide groups, in the cis-configuration, were linked through hydrogen bonds[56]. This has not been confirmed[57], and recent evidence[18,19,54], indicates that the occurrence of two (or more) NH stretching frequencies may arise from a coupling of the vibrations in different amide groups, through hydrogen bonds. This absorption band is sensitive to changes in structure[16,17,54] and may well be a complex band in which the individual peaks are not resolved in the case of proteins.

The frequencies of both the C=O stretching (d) and N—H deformation (e) absorptions are sensitive to changes in structure. Both absorption frequencies are shifted in the α—β transformation[19,66], so that observation of these frequencies affords possibilities in distinguishing α and β phases in amorphous materials[13,43,46,53,55,66].

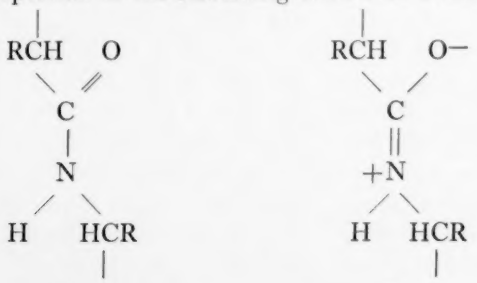
Investigations of the infra-red dichroism in oriented protein structures have shown that in the α-configuration the C=O and N—H groups have

a preferred orientation parallel to the fibre axis, whilst they are perpendicular in the β-configuration[13,16,50,52,66]. The dichroism exhibited by collagen[13,15,49] is similar to that of the β-configuration, but the correspondence does not extend to the overtone and combination bands[13]. These observations, in conjunction with X-ray data and similar measurements on synthetic polypeptides[19,52,54], have been used in attempts to predict the spatial arrangement of the polypeptide chains in the α and β configurations[16,58,66] and in collagen[13,58,71].

Recent investigations by W. C. PRICE and the author[69,70] indicate that considerable caution must be exercised in the interpretation of infra-red dichroism. It has been customary to assume that the transition moment associated with a vibration is along or perpendicular to the bond in question, depending on whether the assignment is to a stretching or bending vibration. In the case of the peptide group, approximate calculations indicate that the 'C = O stretching' and 'N—H bending' vibrations involve significant amplitudes of vibration of atoms other than the bonded pair.

The stretching force constants of the C = O and C — N bonds, calculated from the bond lengths given by PAULING *et al.*[21], are of similar magnitude, and the mechanical interaction between these stretching vibrations and the N — H bending vibration results in three in-plane modes involving motions, to some extent of all four atoms. The direction of transition moment in the 1,650 cm^{-1} absorption band of proteins is not, for this reason, exactly in the CO direction, but is predicted to be inclined at a small angle, veering towards the NO direction.

A second and probably more important contribution to the error in assuming the transition moment to be in the CO direction for this vibration results from the orbit following that must occur. During vibration a change in the proportion of the resonating structures 1 and 2



1. 2.

contributes an additional oscillating dipole moment in the direction NO. The deviation from the CO direction is of the same sense as that predicted by considering the mechanical form of the vibrations. The additional moment also explains the high extinction coefficient of this vibration.

Nucleic acids

The main features observed in the spectra of both ribose and deoxyribose nucleic acids[36,59,60,61,62] are depicted diagrammatically in Figure 2*b*. Absorption band *a*, which is complex, corresponds to OH, NH and NH_2 stretching modes which overlap to a considerable extent. With high spectral resolution certain components of this band may be identified in DNA.[59]. Absorption *b* arises from CH stretching modes. Between 1,600 and 1,800 cm.$^{-1}$ considerable overlapping of the double-bond (C=C, C=N, C=O) vibrations in the purine and pyrimidine bases occurs, leading to an absorption band (*c*) which exhibits some structure. The rest of the spectrum is dominated by the absorptions of the phosphate groups. Band *d* is of interest as it is very much weaker in the spectra of the sodium salts of isolated nucleotides[45,62]. The assignment to a P=O stretching vibration[59] is difficult to reconcile with the equivalence of the two P—O^- bonds which would be expected in the sodium salt of nucleic acid. It may, in fact, be that the vibrations at *ca.* 1,050 and 967 cm.$^{-1}$ in the sodium salt of D.N.A. correspond to the antisymmetric and symmetric vibrations of the $>P\begin{matrix}O^-\\O^-\end{matrix}$ grouping, as in simpler phosphates. Vibrations of the C—O—C groups in the sugar residues and OH bending vibrations (in the case of R.N.A.) will also be expected to contribute to the absorption band *e*. The spectrum between 800 and 1,000 cm.$^{-1}$ (*f*) is characteristic of the particular nucleic acid[60,62], and has been used in the detection and estimation of nucleic acids in tissues[63].

Investigations of the infra-red dichroism exhibited by oriented sheets of DNA.[59] establish a preferred direction for the base residues perpendicular to the fibre axis; deductions from the dichroism exhibited by absorption bands arising from the phosphate and sugar residues are hampered by uncertainties in the assignment of frequencies and directions of transition moment[59,62]. Interesting, but as yet uninterpreted, changes in dichroism occur when these films are rolled or stretched[59].

Dichroism has also been detected in the nucleoprotein of oriented Sepia spermatozoa; the sense of the dichroism is essentially that observed in D.N.A. sheets, except that an additional peak at *ca.* 1,530 cm.$^{-1}$ exhibits parallel dichroism. This corresponds to the NH bending mode in fibrous proteins; the parallel dichroism indicating a β-configuration. Whilst this may indicate an extended configuration for the protein component of the nucleoprotein, the observation is complicated by the unknown contributions of the structural proteins to the observed dichroism.

In the case of tobacco mosaic virus[72], examined as a dried smear, absorption bands at 3,300, 3,060 and 1,656 cm^{-1} exhibit perpendicular dichroism and 1,545 cm^{-1} parallel dichroism with respect to the direction

of shear. The observed dichroisms would be consistent with either β-chains in the protein component oriented in the direction of shear or α-chains perpendicular to this direction. The frequencies 1,656 and 1,545 cm.$^{-1}$ are however those normally associated with the α-configuration[66], and it seems likely that the major part of the protein in T.M.V. exists in the folded configuration with the chain axes having a preferred direction perpendicular to the length of the particle.

Polysaccharides

The absorption spectra of polysaccharides (Figure 2*c*) are less well-defined than with proteins and nucleic acids. Band *a* arises from hydrogen bonded OH stretching vibrations and is generally broad. When amino sugars are present this band is overlapped by the NH and NH_2 stretching modes, and the second NH stretching frequency (*b*) of proteins is observed. The CH absorption (*c*) is similar to that found in 2*a* and 2*b*. When C=O and N—H groups are present bands *d* and *e* corresponding to those of Figure 2*a* are observed. The main feature below 1,500 cm.$^{-1}$, which appears to be common to all polysaccharides, is a strong broad absorption near 1,100 cm.$^{-1}$ (*f*). This originates from vibrational modes of the glucosidic C—O—C linkages, OH bending vibrations, and C—O stretching vibrations.

Measurements of infra-red dichroism have been reported for cellulose and chitin[64]. Again, a detailed interpretation of the dichroism was hampered by uncertainties in the assignment of frequencies and transition moment directions; but, taken in conjunction with X-ray data, considerable progress was achieved in predicting the detailed structure of chitin.

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SOME PHYSICO-CHEMICAL STUDIES ON VIRUSES

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INTRODUCTION

PHYSICO-CHEMICAL techniques have been applied to the study of viruses for a number of reasons. When viruses were first recognised as a cause of disease towards the end of the last century, it was as a result of a physico-chemical method, namely filtration, which was later to be developed as a major technique of the virus worker by BECHHOLD[1], by ELFORD[2], and by others, but which is now obsolete. In the early days of virus work little was known about viruses, except that they were very small, and much of the information about them, particularly as to their size, was acquired by indirect methods. One of these has already been cited; another involves the use of centrifuges for the determination of particle size (BECHHOLD and SCHLESINGER[3]); and others, the effects of ionising radiations (LEA[4]), the use of X-ray diffraction (BERNAL and FANKUCHEN[5] and many other methods (for a review see MARKHAM, LEA and SMITH[6]).

The development of the electron microscope and its application to viruses (KRAUSE[7]; KAUSCHE, PFANKUCH and RUSKA[8]) has largely changed the approach of the virus workers to this problem, and indirect methods are now used to obtain another kind of information such as the presence or absence of water of solvation in the particles. At the same time, and for the same reason, a different type of research has become more common, that using the viruses as convenient model substances for checking the validity of various physico-chemical theories, and it is extremely difficult for someone unfamiliar with the literature to notice where the borderline is drawn. For example the tobacco mosaic virus is used in laboratories all over the world as an easily obtained suspension of rod-shaped particles for which the size distribution can easily be obtained. Much of the work done on this virus is of interest to the virus worker, but a substantial amount might just as well have been done on suspensions of synthetic fibres if these had been available with such uniform diameters. The viruses do, however, provide extremely useful material for this type of work since they may be obtained in different shapes and sizes, though I should point out that many of these substances are extremely difficult to

prepare in adequate quantities for the study of their own properties.

The third field in which physico-chemical methods are of the greatest use is the isolation and purification of viruses, and it is of interest to note here that much of the impetus for the designing of the air-driven ultracentrifuge came from the need for such an instrument for virus research.

In the following survey of physico-chemical work on viruses it is not possible to cover all the important techniques which have been employed in the investigations. For example the work on the effect of ionising and other radiations on viruses requires a complete review of its own (but see LEA[4]), while the electrophoretic method so useful in many protein studies has not yielded information other than of routine interest to virus workers, apart from its biochemical implications.

CENTRIFUGE TECHNIQUES

The centrifuge has always been an instrument of the greatest importance to virus workers, as, besides enabling the isolation of many viruses with the least possible degradation, it may be used in many other ways for finding out information about the nature of the viruses themselves. Much of the early work was carried out with standard laboratory centrifuges but from time to time apparatus has been modified or designed specially for the object of studying viruses.

As early as 1922, two American research workers, MACCALLUM and OPPENHEIMER[9], used a centrifuge technique for purifying the elementary bodies of the Vaccinia virus, and gave the name to the process now known as differential centrifugation, although the method which they in fact used was a different one from the one called by that name nowadays. They centrifuged their virus suspensions in solutions of glycerol having varying densities and chose a solution in which the virus neither sedimented nor rose to the surface. The disadvantages of a method of this type are obvious since the viscosity of the solutions is increased greatly by the glycerol (or sucrose); while the separation of particles differing but little in density becomes a matter of difficulty. This method has only limited utility, but the principle of varying the density of the suspending medium has been widely used for the determination of the specific gravity (ρ) of virus particles.

Some aspects of the theory of centrifugal sedimentation

In an ultracentrifuge, that is a centrifuge in which sedimentation is unhindered by mechanical vibrations, convection and the geometry of the tube, a virus having a sedimentation constant,

$$s = dx/dt\big/\omega^2 x, \qquad \ldots . \ (1)$$

where x is the radial distance of the virus particle from the axis of

rotation, t is the time and ω the radial velocity of the centrifuge, will sediment with a steadily increasing velocity from its original position in the fluid until it hits the bottom of the tube, the time taken being given by the above expression. Thus, in an ultracentrifuge it is possible to calculate the time required to clear a solution of virus of known s (ignoring diffusion effects, which in most cases are very small) taking the case of those particles which are farthest from the bottom of the tube.

In all other types of centrifuge, because of the shape of the tubes and their positioning, and because of vibration and temperature changes, this simple equation does not apply. The extent to which the results deviate from those anticipated from the sedimentation constant does however depend to a large extent upon the concentration of the virus suspension. When a suspension sediments, a boundary forms between the suspending medium, or solvent, and the suspension. If this boundary is accompanied, as it usually is, by an appreciable density gradient, then it is stable, but only so long as the other factors involved, such as convection, are unable to upset this stability. If the suspension is concentrated, it will have a very stable boundary, while if it is very weak, no boundary will be able to form. This last state is the extreme case and has been worked out theoretically by BECHHOLD and SCHLESINGER[3]. Their reasoning is as follows : in a centrifuge tube where the bulk of the liquid is stirred continually to prevent a boundary from forming, only those particles which are near the bottom will have a chance to hit it and be trapped (the trapping is usually assisted by providing a finely-pored plate or a piece of filter paper, inside which convection is suppressed by viscous effects). Consequently the rate at which particles accumulate at the bottom of the tube is a function of the concentration of the solution, which therefore falls off exponentially with time. With the ordinary geometry of centrifuges this rate is much smaller than that in ultracentrifuge sedimentation, so that a stirred centrifuge is relatively inefficient. The only common example of this type of centrifuge is the Sharples Supercentrifuge, and for this machine the theory of BECHHOLD and SCHLESINGER may be applied with some confidence. MARKHAM[10] gives the following general equation for solving the rate of sedimentation in convecting centrifuges :

$$C_t/C_o = e^{-rKAt/V} \qquad \ldots . (2)$$

where C_t and C_o are the concentrations at time t and time o respectively, r is the radius at the *bottom* of the tube and A is the area onto which sedimentation is taking place; $K = \omega^2 s$ and V is the total volume of liquid in the tube. (In the case of the Sharples centrifuge, V may include a large external volume of liquid which is being circulated rapidly through the bowl, out, and round again.) This relationship for 200 ml. of a suspension of a virus having a sedimentation constant of 100 Svedbergs

(100 S), in a Sharples centrifuge running at 40,000 r.p.m. is given in Figure 1.

In centrifuges other than the Sharples, it is difficult to calculate actual sedimentation rates, although with machines having trunnion cups equation 2 will be found to give a close approximation in the case of dilute solutions. With stronger solutions and with angle-heads the problem becomes practically insoluble, and then the time required to effect sedimentation must be determined by experiment. In some modern high

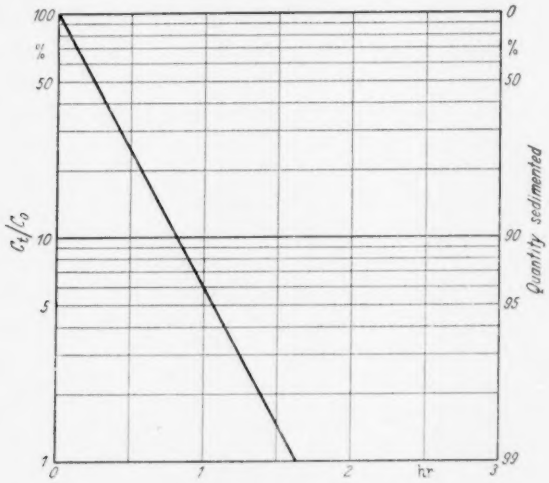

Figure 1. The approximate rate of sedimentation of a virus of sedimentation constant 100 Svedbergs in a Sharples centrifuge running at 40,000 r.p.m.

speed centrifuges, it is possible by using solutions of 2 per cent or more by weight to get quite good boundaries formed, and thus to effect separations which might otherwise be impossible. Figure 2 shows a case in which this has been achieved. Turnip yellow mosaic virus solutions on purification (MARKHAM and SMITH[11]), contain two proteins, having sedimentation constants of about 49 and 106 Svedberg units respectively (Figure 3). When a solution of the correct strength is centrifuged at 12,000 r.p.m. for 2 hours (the optimum time), two boundaries can be seen. The supernatant liquid contains some material (but very little), the next layer is largely (about 90 per cent) the 49 S component and the bottom layer is of course a mixture of the 49 S and 106 S components. The middle layer may be removed after stopping the centrifuge, by means of a pipette, without disturbing the boundary. If the process is repeated several times a stage is reached when the top boundary no longer forms because it is no longer

sufficiently concentrated, and then much of the 49 S component is left in the supernatant liquid, since its sedimentation rate is now approximately as given by equation 2. The pellet at the bottom of the tube then very largely consists of the 106 S component. Advantage may be taken of this phenomenon to effect a practically complete separation of a substance having a *low* sedimentation constant from one of higher sedimentation constant (Figure 4).

It is of some interest to discuss the opposite case *i.e.* that in which the substance with the higher sedimentation constant (X) is too weak to form a boundary while that with the lower s (Y) is strong. In this case

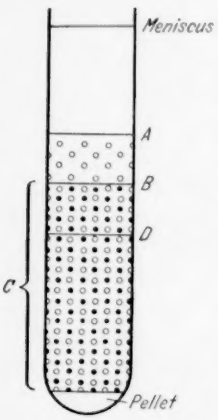

Figure 5. Diagrammatic representation of a centrifuge tube containing components A, B, C and D in order of their sedimentation constants. A, B and D are in sufficient concentration to form boundaries while C is not. In this case the B boundary acts as a liquid ultrafilter membrane carrying down the C component with it.

the concentration of X will increase as the boundary of Y proceeds down the tube, since although X is convecting in the bulk of the solvent, as soon as it enters the boundary of Y it will cease to do so, because of the density gradient there; and as its s is larger than that of Y, it will then sediment more rapidly than the boundary and enter the stirred region again. This process will be repeated until X is concentrated enough to form its own boundary or until the boundary of Y hits the bottom of the tube. In this process the Y boundary acts exactly as if it were a semi-permeable membrane, impermeable to X, but not to the solvent or to any substance having an s less than that of Y. This effect should have considerable application, as in order to concentrate any substance which is present in a solution in very small quantities, it is only necessary to add a large amount of a substance having a lower s and spin the latter down. For example, it is possible to remove the turnip yellow mosaic virus protein of $s = 106$ S, contaminating that of $s = 49$ S, merely by adding *Helix pomatia* haemocyanin of $s = 100$ S, and spinning that boundary through a dilute solution of the other proteins, when it will only carry down the 106 S component, leaving much of the slower component still unsedimented. A diagrammatic representation of the sedimentation of a mixture of four components, one being very weak, is given in Figure 5.

The above short discussion about the sedimentation behaviour of mixtures is of interest because it demonstrates that the fractionation of a mixture by high speed centrifugation may be considerably greater than one would calculate from simple sedimentation theory, and it seems not

unlikely that this kind of fractionation may remove interesting materials without the knowledge of the workers concerned. It is practically certain that the existence of two related substances in plants diseased with the turnip yellow mosaic virus would not have been noticed if a centrifuge technique had been used for the isolation instead of a salt precipitation method.

QUANTITATIVE CENTRIFUGATION MEASUREMENTS

Viruses are exceptionally suited to observation by quantitative centrifugation techniques because of their size. The recorded sedimentation constants range from 50 S to 5000 S units for one of the tobacco necrosis viruses and vaccinia virus respectively. This means that quite low centrifuge speeds are required, and speeds exceeding 24,000 r.p.m. are exceptional. This is a considerable advantage since the rotor temperature may be assessed at this speed with confidence in modern ultracentrifuges, because the temperature rise is exceedingly small, and therefore the solvent viscosity (η) in the cell is known with adequate accuracy. Moreover, as the diffusion constants are small (generally less than $1{\cdot}5 \times 10^{-7}$ cm^2/sec., equivalent to a sphere of about 28 mμ diameter in solution), the boundaries are unusually sharp (Figure 4) and their positions are easily measured, while, as the resolution of two components having similar sedimentation constants is a function of the boundary spreading which takes place, it is possible to resolve mixtures differing only slightly in sedimentation constants.

To counteract these advantages there are some disadvantages. With many ultracentrifuges, the low speeds required for the study of the larger viruses are difficult to maintain, while the sharp diffusion gradients cause such high refractive index gradients that it becomes imperative to use very small concentrations (about 0.2 mg/ml), or thin cells, and consequently the areas and shapes of the refractive index gradient diagram are difficult to measure accurately. Refractive index methods are used almost exclusively nowadays, mainly in the form of the 'diagonal-Schlieren' technique, although the 'scale' method is still in use. Most early measurements were made by the light absorption technique, but this method is now almost obsolete. The light scattered by solutions of the larger viruses tends to obscure some of the information obtained by refractometric techniques, but this may be reduced by the use of red light for making the observations.

In the case of many of the animal viruses and also that of some preparations of the tobacco mosaic virus, the particles are not all uniform in size, and consequently the diffusion boundary in the ultracentrifuge has another distribution due to differences in sedimentation constant super-

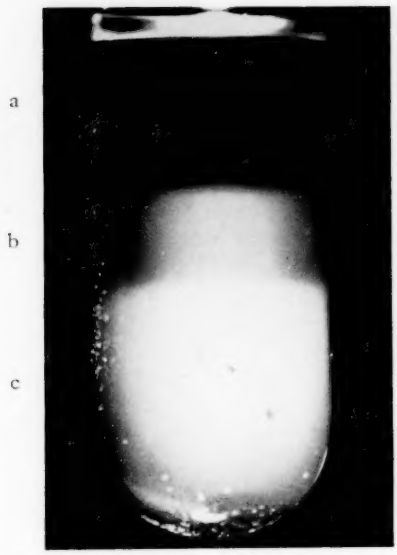

Figure 2. Boundaries formed in a high speed centrifuge showing the two components of turnip yellow mosaic virus preparations. The top layer *a* is the solvent, the middle one *b* the virus protein and the lowest one *c* the virus nucleoprotein plus the virus protein.

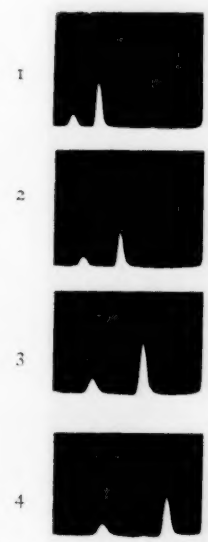

Fig. 3

Fig. 4

Figure 3. A series of diagonal Schlieren diagrams of the mixture of proteins in turnip yellow mosaic virus preparation. The smaller (protein) component constitutes about 25-30% of the total material in most preparations. 24,000 r.p.m., 10 minutes between successive exposures.

Figure 4. Turnip yellow mosaic virus nucleoprotein isolated from a mixture of protein and nucleoprotein by centrifuging. Ultracentrifuge diagonal Schlieren diagram. Speed 24,000 r.p.m. five minutes between successive exposures.

imposed upon it. As the diffusion constant is much less sensitive than the sedimentation constant to differences in particle size, it is possible to use this extra spreading to determine the size distribution of the particles of a virus. The second moment of the refractive index gradient curve may be taken as consisting of the sum of two variances, $\sigma_1^2 + \sigma_2^2$, where $\sigma_1^2 = 2Dt$ (D = the diffusion constant of the virus, obtained by a separate experiment, or in some cases calculated from other data, and t = the time). σ_2^2 may then be determined and is the square of the standard deviation of sedimentation constant for the virus. From this the distribution of radii may be calculated (see, for example, STANLEY and LAUFFER[12]).

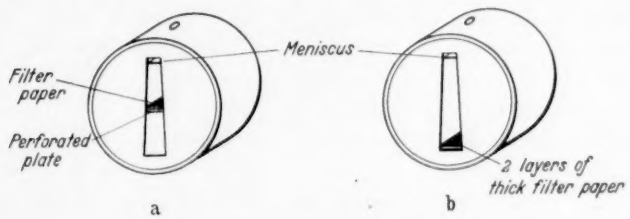

Figure 6. (*a*) The ultracentrifuge separation cell. (*b*) A cell improvised from an ordinary ultracentrifuge cell.

In the case of the bushy stunt virus the fit between calculated and observed diffusion boundaries has been shown to be nearly perfect (LAUFFER[13]).

Besides allowing for the measurement of sedimentation constants to be made, centrifugation also allows the correlation of a physical property with a biological activity, namely infectivity, and thus one may show the identity of isolated material with the virus, as in the case of the tobacco mosaic virus (LAUFFER[14]). Such measurements are usually made with the separation cell of TISELIUS, PEDERSON and SVEDBERG[15] (Figure 6*a*) which contains a perforated plate covered with hardened filterpaper, but we have found it equally satisfactory to place two strips of a thick filterpaper at the bottom of an ordinary cell (Figure 6*b*). In both cases the boundary is driven towards the porous material which prevents the contents of the two parts of the cell from mixing when the centrifuge is stopped. The upper layer is then tested in the appropriate way and the quantity of material correlated with that calculated from the position of the boundary. The cell (*b*) may also be used for small fractionations under optical control.

An analogous technique employing an ordinary laboratory centrifuge has been used by BRAKKE, BLACK and WYCKOFF[16] to determine the approximate sedimentation constant of the potato yellow dwarf virus. These authors centrifuged in capillary tubes and correlated a visible boundary with the infectivity of samples removed by means of a capillary

pipette. The value obtained, namely 1,150 S, is the largest yet reported for a plant virus by a factor of six, and further studies on this virus will be awaited with great interest.

The technique described above is essentially that of ELFORD[17] which has already been discussed critically (MARKHAM *et al.*[6]). In all measurements of this type it must be borne in mind that extremely dilute virus solutions can be estimated by infectivity measurements. Consequently the virus may not be sufficiently concentrated to form a stable sedimenting boundary and may therefore be sedimenting in a convecting manner or may even be coming down with a stable boundary having a lower sedimentation constant, as has already been explained. The convecting centrifuge technique of BECHHOLD and SCHLESINGER[3] is more suitable under these conditions and may give excellent results. It is of interest to note here that the data of these authors for the tobacco mosaic virus when recalculated give a sedimentation constant of 167 S for the virus in crude plant sap (BECHHOLD and SCHLESINGER[18]), which, considering that the measurement was made in dilute plant sap, is in excellent agreement with modern data obtained on purified material ($s = 190$ S), while the infectivity methods used would not be expected to detect much less than tenfold differences in concentration.

The interpretation of ultracentrifuge measurements is complicated by factors which have no easy solution. In the case of the plant viruses and a few others, the results are probably best expressed as 'molecular weights' (the weight in grams of $N = 6.023 \times 10^{23}$ particles) by means of the usual Svedberg equation :

$$M_0 = \frac{RTs_{20}}{D_{20}(1 - V_0\rho)} \qquad \ldots . (3)$$

where M_0 is the dry molecular weight, $T = 293°\text{K}$, s_{20} is the sedimentation constant and D_{20} the diffusion constant in absolute units at 20°C respectively; V_0 is the specific volume of the dry virus and ρ is the density of water at 20°C (= 0.998). R is the gas constant ($= 8.314 \times 10^7$ erg. deg^{-1} mole^{-1}). V_0 is easy to determine if sufficient material is available, which is rarely the case, while in most cases D is so small that it is subject to extraneous errors of considerable magnitude. For the smaller plant viruses these difficulties may be overcome and adequate values for M_0 have been obtained. A diffusion diagram for the turnip yellow mosaic virus is shown in Figure 7.

Methods for the calculation of the various constants of viruses have been discussed in detail by MARKHAM *et al.*[6] and it is unnecessary to reproduce here all the formulae given in this paper (which is reproduced by SMITH[19]). A few formulae are given here merely to indicate methods, and others are intended to supplement this paper.

The most usual way to express sedimentation data is by means of the diameter ($2r$) of the spherical particles in solution (or the average diameter of near spheres). This is most conveniently accomplished by the use of the formula:

$$2r = 0{\cdot}426 \left(\frac{Vs_{20}}{1 - V\rho} \right) \quad \ldots . (4)$$

which is based on STOKES' law. V is the volume occupied *in solution* by 1 g of virus. V is always larger than V_0 and is rather difficult to determine. Viruses, like proteins, take up water when in solution and this makes them larger. The value for this increase is easily obtained if D is known, but in many cases it is not. Consequently methods have been worked out for determining V indirectly. Perhaps the most widely used one is to assume that the water associated with the particles does not act as a solvent for other substances. Consequently the buoyancy of the solvent may be increased by adding salts or sugars, and measuring either the decrease in s, or the density of solvent at which $s = 0$. This technique is full of doubtful assumptions, since the apparent V may in many cases vary with the concentration of the added material. This is particularly true if the solvent density is increased by adding heavy water, as this equilibrates with all the water and some of the hydrogen atoms in the virus. Hence this method may be used to give an approximate value of V_0. Perhaps the best substances for increasing solvent densities are the proteins, provided always that no interaction takes place between the protein and the virus (SHARP, TAYLOR and BEARD[20]). SCHACHMAN and LAUFFER[21] have pointed out that in the case of small viruses like the tobacco mosaic virus the dimensions of protein molecules are such that one cannot assume contact of the latter over the whole of the former, and consequently a value of V may be obtained which is much too large. The values of V obtained seem to range from about 0.8—0.9, showing that a large amount of water is bound by the viruses in solution. V_0 for most proteins is about 0.75, while that for many viruses is smaller on account of their nucleic acid content.

An alternative method for determining V or its equivalent for spherical viruses is to measure their viscosity increments. This method is only suitable for those cases in which viscous impurities can be excluded. Thus, for example, it cannot be applied with certainty to influenza virus suspensions, as these often have a viscous contaminant (STANLEY and LAUFFER[12]). EINSTEIN[22] showed that for a suspension of spheres:

$$\eta_{rel} - 1 = 2{\cdot}5\phi \quad \ldots . (5)$$

where ϕ is the volume fraction of the solution occupied by spheres and η_{rel} is the viscosity of the solution relative to that of the solvent. It is thus a

simple matter to calculate V if V_0 is known. An alternative and rather more elegant method of calculation is as follows. Defining the intrinsic viscosity of the virus as $\eta_{rel} - 1$/g *dry* wt./ml. $= G$, we get:

$$2r = \left(\frac{36\eta sG}{5\,(1 - V_0\rho)}\right)^{\frac{1}{3}} = 0{\cdot}2695 \left(\frac{Gs_{20}}{1 - V_0\rho}\right) \quad \ldots . (6)$$

and the *dry* molecular weight:

$$M_0 = 3{\cdot}6\, N\,(5G)^{\frac{1}{3}} \left(\frac{\eta\, s}{1 - V\rho}\right)^{3/2}$$

$$= 1{\cdot}543 \times 10^{22} G^{\frac{1}{3}} \left(\frac{s_{20}}{1 - V_0\rho}\right)^{3/2} \quad \ldots . (7)$$

$$\text{and } V = \frac{0{\cdot}4\, G}{(1 - V_0\rho) + 0{\cdot}4\rho G} \quad \ldots . (8)$$

For the bushy stunt virus, $G = 3{\cdot}44 \pm 0{\cdot}13$ (unpublished), $s_{20} = 131{\cdot}5$ and $V_0 = 0{\cdot}734$.

Hence $2r = 35{\cdot}1$ mμ and $M_0 = 9{\cdot}9 \times 10^6$ and $V = 0{\cdot}839$.
These calculations may be compared with values obtained by other methods (MARKHAM *et al.*[6]), $2r = 37{\cdot}1 \pm 3{\cdot}8m\mu$, $M_0 = 10{\cdot}65 \times 10^6$, $V = 0{\cdot}853$, for which calculation a slightly different value for V_0 was used (0·739).

A similar type of calculation has been used earlier by MARKHAM *et al.*[23] to show that suspensions of the rabbit papilloma virus obeyed EINSTEIN'S viscosity law. Using the viscosity value together with the latest values for the other constants of this virus (SHARP, TAYLOR and BEARD[20]), we get $V = 0{\cdot}90$, compared with 0·887 obtained by these authors by centrifuging in bovine serum albumin solutions.

The calculation from such data of the dry particle size, which is of course what one sees on the electron microscope, is complicated by the fact that one must assume that the particles shrink to form a solid sphere of density $1/V_0$. That this is not necessarily so is shown by the results obtained on the protein component of the turnip yellow mosaic virus by BERNAL and CARLISLE[24]. In this protein, it appears that an open structure persists on drying[25] and this throws doubt on earlier estimates of dry particle sizes obtained by indirect methods.

Particularly in the case of the elongated viruses like tobacco mosaic, considerable dependence of s against concentration is to be found and it is usual in these cases to extrapolate s to zero concentration. LAUFFER[26] has attempted to correct for this factor by an empirical observation, that, if the sedimentation constant of tobacco mosaic virus is

multiplied by the relative viscosity of the solution, a constant value is obtained which is equal to s extrapolated to zero concentration, and he suggests that this method should be employed in all work. While this result seems to be in accordance with much experimental data, it seems rather unlikely that the viscosity of the solution is itself the causal factor, but rather some other factor which is itself also concentration dependent.

The possibility of the orientation of asymmetrical virus particles during sedimentation has been discussed by various authors (*e.g.* PUTNAM[27]), but a little reflection will show that the rates of movement of particles in an ultracentrifuge are very small indeed, and one would not be likely to get conditions where orientation is an important factor.

Some viruses, notably tobacco mosaic, some bacteriophages, and insect polyhedral viruses show more than one ultracentrifuge boundary under some or all conditions. This is usually attributed to the formation of di- and polymers in solution. In the case of bacteriophages, two boundaries are given in acid solution, (SHARP, HOOK, TAYLOR, BEARD and BEARD[28]), the sedimentation constants being 700 and 1,000 S, for what are probably single and double particles respectively. In tobacco mosaic virus the particles readily polymerise and a component generally considered as a dimer is frequently present in preparations. In the case of the polyhedral viruses, many occur naturally as bundles of rods, which break down to numbers of one, two or more particles packed side by side, and in this case several sedimenting boundaries can be seen.

MICROSCOPICAL METHODS

The light microscope has definite limitations in the study of individual virus particles because of the limits of resolution of this instrument, though the ultraviolet microscope can resolve most of the larger viruses. For example BARNARD[29] claimed a resolution of 75mμ for his equipment and this is adequate to resolve the larger bacteriophages, influenza virus, etc. Many viruses are easily seen by dark-ground illumination and it is possible for example, to count the larger bacteriophages by this means. A most interesting demonstration by this technique is the release of the polyhedral viruses of insects from the crystalline inclusions or polyhedra associated with these diseases by dilute alkali, when the virus particles may be seen in violent Brownian movement inside the skin which forms round each crystal (BERGOLD[30]). Observation of this kind of phenomenon should be illuminating to those who think that wet object mounts for the electron microscope would give more information than the usual dry ones, as the magnitude of the Brownian movement is larger than is usually realised.

Many attempts have been made to extend the use of the light microscope for virus work by the use of fluorescent stains (HAGEMANN[31]) which give an effect similar to dark-ground illumination, or intensification of the particles by techniques similar to those used in photography (BECHHOLD and VILLA[32]), but for the observation of individual particles the light microscope has now been completely superseded by the electron microscope. It cannot, however, be too highly stressed that tissues are full of particles of the same size and appearance as viruses, and that mere observation of 'virus particles' by any microscopical method is insufficient identification. It is true that in certain cases, such as that of the large bacteriophages, it is possible to correlate the number of particles seen with the infectivity of the suspension. In many recent publications it seems unlikely that the objects referred to as virus particles have any relationship with viruses, while in others the wrong virus has actually been photographed. This is particularly the case with the tobacco mosaic virus which is always liable to be present in tobacco and tomato plants.

Although the viruses are all within the limits of resolution of the electron microscope, their electron scattering ability is so small that the contrast obtained is hardly sufficient for visual observation, and the metal shadowing technique of WILLIAMS and WYCKOFF[33] is now almost universally used. The granulation observed in early specimens, which has occasionally been misinterpreted as structure (MANDLE[34]), has been overcome by the use of refined techniques and the avoidance of metals such as pure gold, which have a tendency to granulate. Metal shadowing inevitably makes the specimens larger and the apparent increase in size of small viruses due to the metal layer may be an appreciable percentage of their diameters.

Metal shadowing techniques frequently mask internal structures which are present, and there are cases where unshadowed specimens have shown details which cannot be demonstrated by shadowing. One instance is the crystallisation of the turnip yellow mosaic virus which is discussed elsewhere, while another example of extreme interest is the work of ANDERSON on bacteriophage (1951)[35]. In this research, a technique of dehydration and drying of specimens has been worked out involving the replacement of the water in the specimen by a liquid miscible with liquid carbon dioxide, and finally by the latter liquid, which has a critical point of 31·1°C. On exposing the specimen in liquid CO_2 to a higher temperature than this, the carbon dioxide becomes a vapour, no interface being formed, so that specimens may be prepared and dried without the distorting effects of surface tension. When prepared in this way, specimens of *Escherichia coli* which have had the T_2 bacteriophage adsorbed on to them, resemble pincushions, the 'phage particles being attached by the tips of their tails to the bacteria. This is a remarkable illustration of the use of this technique,

and also demonstrates the function of the tails on these viruses. The 'phage particles in solution are of course rotating all the time under Brownian movement, and as the centre of gravity of the virus is situated inside the head the tail sweeps out spheres of much greater volume than that of the head, and consequently the chances of the 'phage hitting a receptor spot on a bacterium are much greater than if the 'phage were spherical without a tail. Not all 'phages are tailed, but the presence of a tail would appear to be of definite advantage to 'phages like T_2.

The main use of the electron microscope in the observation of viruses has been in determining their shapes and internal structures rather than their absolute sizes, and much of the work done has confirmed results obtained by other methods. For example, the general appearance of the tobacco mosaic virus was known some years before it was photographed on the electron microscope by KAUSCHE, PFANKUCH and RUSKA[8] because of the X-ray crystallographic work of BERNAL and his associates and the observations of TAKAHASHI and RAWLINS[36] and others on the flow birefringence of the solutions of the virus. On the other hand the mere observation of the *shape* of the virus particles has been of considerable importance in some instances. An example of this is the case of the Shope rabbit papilloma virus, which had been submitted to examination by ultracentrifugation, diffusion and viscosity measurements by NEURATH, COOPER, SHARP, TAYLOR, BEARD and BEARD[37] who deduced from their data that the virus was probably a flattened cylinder with an axial ratio of about 7:1. Electron micrographs obtained by SHARP, TAYLOR, BEARD and BEARD[38] showed that the virus particles were in fact spherical or nearly so, and MARKHAM, LEA and SMITH[23] pointed out that the experimental data were in fact consistent with the real shape of the virus.

Measurements of the size of particles made by means of the electron microscope are inevitably liable to some uncertainty, due to the fact that the magnification calibration of these instruments is usually subject to some error, and no completely satisfactory method for overcoming this has yet been found. Calibrated uniform suspensions of polystyrene have been used as internal standards in several cases, but it seems doubtful whether they are as uniform as has been claimed, while at the other extreme, the repeat structure of such objects as diatoms has been used for calibration, a procedure which would appear to be a return to the most primitive type of micrometry. Fortunately it is now well known that only the few crystalline plant viruses can be regarded as being regular and uniform in their sizes, while many other viruses have now been shown to have large size distributions. Many of these also show signs of internal organisation which are more reminiscent of bacteria than of molecules, so that for many purposes extreme accuracy of measurement is not as

important as the observation of morphological detail, which is readily obtainable by normal techniques.

A different approach to the problem has been made by WILLIAMS and BACKUS[39] who sprayed fine droplets of tomato bushy stunt virus suspensions of known concentration also containing known weights of polystyrene latex onto microscope grids. The spots formed were 5-30μ in diameter, and in such areas the total number of latex particles was counted and also the number of virus particles. From this ratio, which averaged 50 latex spheres to 100 virus particles, they calculated the molecular weight of the virus, which was found to be $9{\cdot}4 \pm 0{\cdot}7 \times 10^6$, which is in excellent agreement with other methods. All reliable values for this constant are given in the accompanying table.

Table I. The molecular weight of the bushy stunt virus

$M_0 \times 10.^6$	*Data on which measurements are based*	*Authors*
$9{\cdot}4 \pm 0{\cdot}7$	Direct count of particles	WILLIAMS and BACKUS[39]
$10{\cdot}65 \pm 1{\cdot}1$	s_{20}, D_{20}, V_0	NEURATH and COOPER[40] LAUFFER and STANLEY[41]
10·65	Crystal lattice plus crystal density	BERNAL, FANKUCHEN and RILEY[42] calculated by MARKHAM *et al.*[6]
$9{\cdot}90 \pm 0{\cdot}5$	Solution viscosity, s and V_0	This article
9·0	Light scattering	OSTER[43]

THE STUDY OF VIRUS CRYSTALS

The successful production by STANLEY[44] of tobacco mosaic virus in a para-crystalline form, and the crystallisation by BAWDEN and PIRIE of the bushy stunt virus of tomato[45], and the subsequent crystallisation of several plant viruses has opened a field of investigation which was quite unexpected in the earlier day of virus research.

Tobacco mosaic

Tobacco mosaic virus when purified will deposit a mass of fine needles from solutions when acid or salt is added to them. These are the para-crystals of the virus. Similar but rather plumper bodies, or tactoids, form in concentrated solutions of the virus (BAWDEN and PIRIE[46]), and, being denser than the bulk of the solution, settle down to the bottom of the container and there form a layer known as 'bottom layer' which is stable. This bottom layer is birefringent, but without extinction directions *i.e.* it behaves as if it were a powder of a birefringent crystal. Just what the forces are which cause the formation and maintenance of these tactoids is at present uncertain. The bottom layer material seen under the microscope is striated, and when it is looked at in polarised light is seen to consist of birefringent areas which may be orientated by flow (Figure 8).

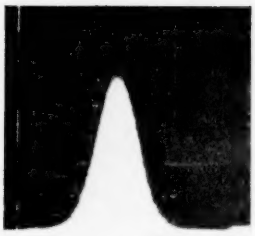

Figure 7. Diffusion of a 1% solution of the turnip yellow mosaic virus at *p*H 7 for 15·72 × 10^4 secs. D_{20} = 1·55 × 10^{-7} cm^2 sec^{-1}. M_0 = 5 × 10^6. Diagonal Schlieren pattern.

Figure 8. 'Bottom layer' of tobacco mosaic virus seen under the polarising microscope. Low power.

Figure 10. Hair of tobacco leaf showing the birefringent plates of tobacco mosaic virus in the top cell. Polarised light. The two transverse bands are cell walls.

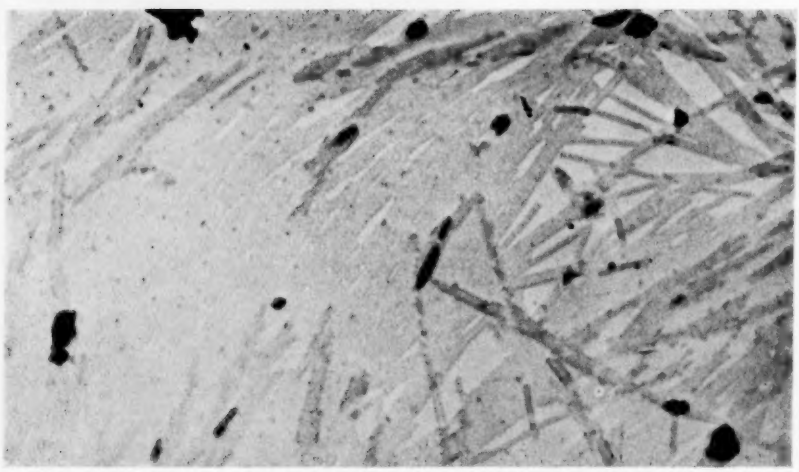

Figure 11. Tobacco mosaic virus. Unshadowed preparation showing side-by-side aggregation of the particles. Magnification about 43,000.

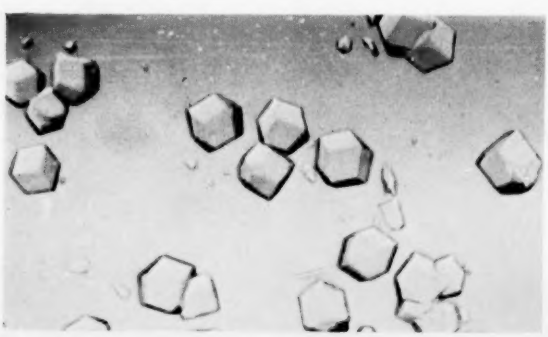

Figure 12. Rhombic dodecahedra of the bushy stunt virus. × 500.

At higher shear rates, such as may be applied by shearing the solution between glass or quartz plates, the orientation becomes complete, and it is possible by this method to prepare thin films of completely orientated virus for optical investigations. Bottom layer may also be orientated by drawing it into a capillary tube, but the most interesting technique from a practical point of view is that employed by BERNAL and FANKUCHEN[47]. In this method a thin cell is formed between a cover glass and a microscope slide, by means of two glass threads. The top and bottom of the cell are open and the bottom layer solution, held in by surface tension, evaporates more rapidly at the bottom edge of the cell. Gradually the whole solution dries out, leaving an orientated dry paracrystal or 'dry gel' which may then be examined by x-ray diffraction or polarised light microscopy, or by electron microscopy (WYCKOFF[48]).

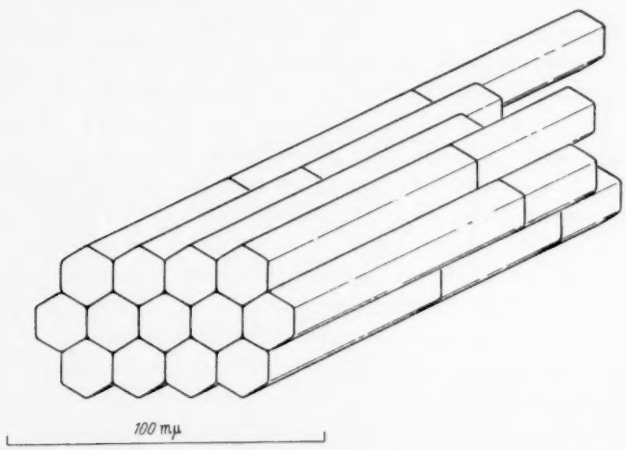

Figure 9. Diagrammatic representation of the structure of the 'dry gel' of the tobacco mosaic virus.

Following the transition from bottom layer, through the stage of 'wet gel', which is a hydrated form of the dry gel, it was found by x-ray diffraction methods that the virus rods became arranged in two dimensions in a regular hexagonal array (Figure 9) even when the interparticle distance is several times the diameter of the particle. When the dry gel is completely dry the interparticle distance is 15 mμ. What the actual shape of the cross section of the particles is is uncertain, but it would seem reasonable to suppose that it is intermediate between hexagonal and circular. The existence of this type of packing in the dry and wet gels is very interesting, as for years it has been known that tobacco and other plants diseased with this virus have in their cells hexagonal plates which are associated with the disease (Figure 10), and for some time it has been

known that these crystals do in fact contain virus. Some workers have claimed that they have managed to crystallise tobacco mosaic virus from solutions in the form of real crystals (*e.g.* KAUSCHE[49]), but no really convincing evidence of this has yet been produced. From their X-ray studies, BERNAL and FANKUCHEN[47] have deduced that each virus rod has a repeat structure of 6·8 mμ along its length, and that the whole rod may be considered as a crystalline unit of fixed diameter but indefinite length. It would seem likely therefore that in the formation of wet gel a random packing in the particle length would take place, as there is no obvious mechanism for keeping the ends of the rods opposite each other, which would be necessary for true crystals to be formed, while in most cases the rods are not of uniform length, because end to end aggregation takes place readily when the solutions are subjected to any of a large number of treatments. WILKINS, STOKES, SEEDS and OSTER[50] have examined the intracellular crystals in ultraviolet and polarised light, and there seems little doubt that the major component of these crystals is in fact a substance having most of the properties of the virus. Many of these crystals seen approximately normal to the hexagonal axis exhibit two series of extinction positions at a small angle to each other, forming narrow bands *i.e.* are multiple layer twins, and the distance between these layers is of the order reported by many American workers for the "natural" length of the particles *i.e.* about 300 mμ. This work is conclusive evidence of the existence inside plant cells of large numbers of virus particles having identical lengths, though, of course, nobody would suggest that other lengths do not exist, if only during the formation of the rods.

An observation which would appear to be at variance with the X-ray crystallographic work is the demonstration by many workers of the tendency of the tobacco mosaic virus to side-by-side aggregation, which may be observed under the electron microscope. In many preparations sheet formation is to be seen extending for fifty or more particles side by side (Figure 11), and the frequent occurrence of other sheets running at right angles to these would make it seem extremely unlikely that a two dimensional regular hexagonal packing of parallel rods could have existed in the solutions examined. The fact that these sheets can and do exist in the bottom layer solution may be demonstrated by examining frozen dried specimens. The formation of the hexagonal packing would then apparently have to involve a moving of the parallel rods apart and a rearrangement into a hexagonal network as the drying process proceeded.

Bushy stunt virus

This virus crystallises as beautiful rhombic dodecahedra (Figure 12) from cold saturated solutions of the amorphous virus. Like many proteins, particularly the haemoglobins, viruses like the bushy stunt, tobacco

necrosis and (sometimes) turnip yellow mosaic viruses are precipitated first from salt solutions in an amorphous condition. The amorphous precipitate has a high *negative* temperature coefficient of solubility, while the crystalline material does not, so that, on cooling, a suspension of the amorphous material will dissolve and then gradually deposit in a crystalline condition. These crystals are isotropic and belong to the cubic system. X-ray examinations of the wet and dry crystals as a powder have been made by BERNAL, FANKUCHEN and RILEY[42] who showed that the crystals in the wet form had a body centred cubic lattice with a cube edge of 39·4 mμ. On drying, the crystals and the unit cells shrink, the latter to 31·8 mμ (Figure 13). This means that if the particles are spheres in point contact, from the dimensions of the cube, the centres of the wet particles are 34·1 mμ apart and in the dry state only 27·6 mμ. The conception of point contacts existing seems unlikely, and other data obtained by indirect methods suggests that a slight distortion takes place in the crystal lattice, the actual particle size being about 37 mμ wet and 29 mμ dry (MARKHAM, LEA and SMITH[6]).

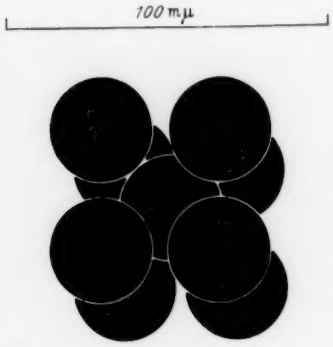

Figure 13. The body centred cubic cell of the bushy stunt virus.

This direct demonstration of the difference in size of dry virus particles and wet ones was of considerable interest, as many physical chemists had endeavoured to reconcile the hydrodynamic behaviour of protein solutions with the masses of the molecules by invoking molecular asymmetry (NEURATH[51]), and although this concept is gradually being abandoned, it is still lingering on in textbooks. Proteins on the whole seem to be either very asymmetrical or nearly spherical, and this also seems to be the case for the simpler viruses.

In the case of the bushy stunt virus, we have calculated that the amount of water associated with the virus is about 0·7—0·8 g of water per g. How it is bound is still a matter for speculation, but it should be pointed out here that in the crystal as well as in aqueous solution, an external layer of water of a thickness comparable with the diameter of a small protein molecule would have to exist as a rigid structure round the particles if it were superficial, and moreover it would itself have to have a configuration giving the immunological specificity of the virus. The obvious inference is that it is held in some kind of internal structure, and that reversible shrinkages and swelling takes place.

The measurements on the powder specimens of this virus have since been confirmed on single crystals by CARLISLE and DORNBERGER[52]

who obtained slightly smaller cell sizes (36·8 mμ wet, 31·4 mμ dry).

Bushy stunt virus has also been prepared by COHEN[53] in other crystalline forms, but these have not been submitted to study. The crystals have not proved suitable for electron microscope observation, possibly because of their rather open structure, but the virus particles themselves show a tendency to form two dimensional sheets of close-packed spheres with a regularity extending for hundreds of particles. Objects like these may well prove excellent standards for the calibration of electron microscopes for magnification.

Tobacco necrosis and Southern bean mosaic viruses

Of all the viruses which have been purified so far tobacco necrosis is by far the most suitable for electron microscope observation. It should possibly be mentioned here that 'tobacco necrosis' is a term covering a number of viruses having similar biological properties, some of which are not related to others. Many of these form fine plate-like crystals, evidently cleaving easily into thin lamellae (Figure 14), which are isotropic when viewed normal to the flat surfaces. When dried down, the plates lie flat, and they may then be shadowed with a suitable metal, such as palladium, and the metallic replica of the surface stripped off on collodion and examined under the electron microscope. In some of the pictures obtained it is possible to see the crystal structure both on the flat faces and on the pyramid edges (Figure 15) (MARKHAM, SMITH and WYCKOFF[54]). This picture shows a strain of tobacco necrosis virus having particles of some 24 mμ in diameter which pack into the crystal in an approximation to cubic close packing. The crystal structure is, of course, not the latter as the crystals are birefringent, and it is impossible to say definitely whether the angles between the rows of molecules on the flat faces are exactly 90° on account of possible distortions produced in making the replicas. Figure 16 gives a diagrammatic representation of part of the electron micrograph, showing the arrangement of the layers of the molecules. In such replicas it is not possible to determine the depth of the layers with much accuracy.

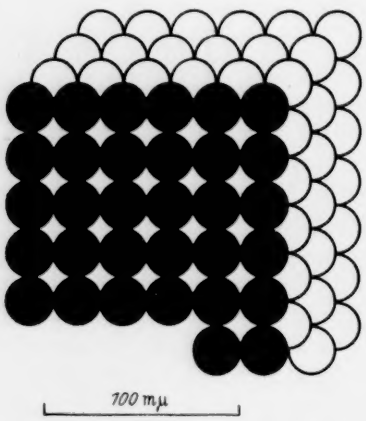

Figure 16. Diagrammatic representation of the molecular arrangement in the tobacco necrosis crystals of Figure 15.

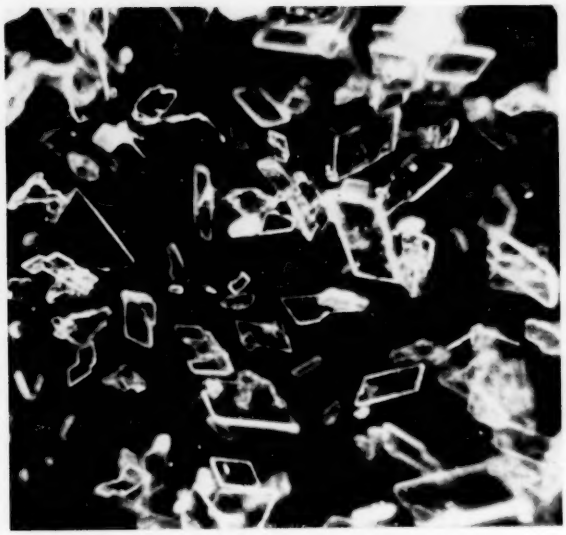

Figure 14. Crystals of a strain of tobacco necrosis virus. Dark ground illumination.

Figure 15. A replica of a tobacco necrosis virus crystal seen under the electron microscope, showing detail on three faces of the crystal.

Figure 17. Crystals of turnip yellow mosaic virus deposited on the wall of a flask.

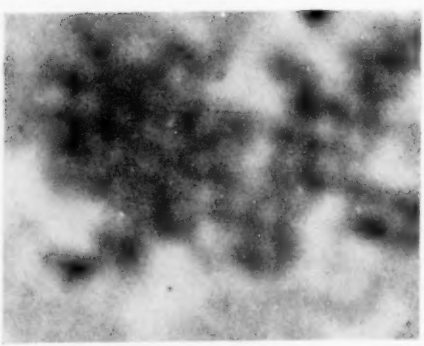

Figure 18. Microcrystalline meshwork deposited from solutions of turnip yellow mosaic virus on electron microscope grids. The darkest regions are several molecules thick. Unshadowed preparation (× 140,000)

A derivative of an unrelated tobacco necrosis virus has had its crystal structure studied by CROWFOOT and SCHMIDT[55]. The unusually large crystal gave highly detailed diffraction patterns on a stationary specimen, but was unfortunately destroyed before the completion of the work. The molecules in the crystal were much smaller than those of the viruses examined by electron microscopy.

The southern bean mosaic virus was the first to be examined by means of surface replicas of its crystals. The arrangement of the particles is not so easily seen in this virus as it is in the tobacco necrosis virus, but a very interesting observation has been made by WYCKOFF[48], and that is that, in the crystal lattice, the virus particles have an axial ratio of about 1·5:1. As the virus particles deposited from solution are apparently spherical, this suggests that a change in shape may take place when larger proteins enter a crystal lattice.

Turnip yellow mosaic virus

This virus crystallises from salt solutions as regular octahedra which frequently have well developed cube faces (Figure 17). These crystals break up under the treatments used in obtaining shadowed replicas, but solutions of the virus dried down in the usual way for electron-microscopic observation frequently give rise to objects looking like pieces of wire mesh (Figure 18), and consisting of electron absorbing material having holes in it arranged in a hexagonal pattern (COSSLETT and MARKHAM[56]). Enough of these have been examined to give a fair idea of their structure (Figure 19). The holes are arranged in rows in three main directions, and the angles in which these directions are arranged with respect to each other are also shown in the figure. The distances between the holes have also been measured in four directions, those indicated by the lines in the figure and also vertically. From diffusion and sedimentation measurements the size of the virus particle was already known with fair certainty and is included in the diagram for comparison.

The regularity of this type of pattern combined with the fact that it evidently also extended in a direction normal to the picture, as can be seen from the density of the pattern, led to the conclusion that these objects were micro-crystals formed on the microscope grids. Now in the cubic system, the only possible open structure having holes in it is the diamond lattice. The constants of that lattice were calculated, taking, instead of the intermolecular distances, the distances between the centres of holes in the plane of twofold symmetry, and these values were compared with the figures obtained from the electron micrographs, the latter being orientated quite readily, even when only a few holes were visible, by the angular relationships of the rows. This procedure is strictly analogous to crystal structure determinations from x-ray diffraction measurements. The

best series of values have been obtained from a single large micro-crystal of the 'top' or protein component of this virus in which the distances between 104 pairs of holes were measured and the weighted mean diameter

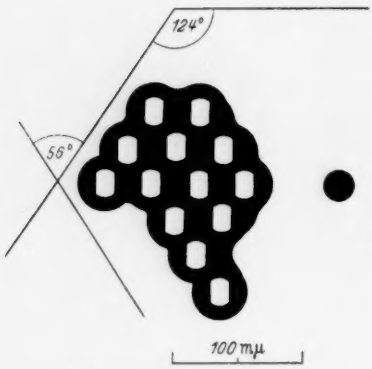

Figure 19. Enlarged and idealised diagram of the microcrystalline deposit of the turnip yellow mosaic virus. A single particle of the virus is indicated on the right to give an idea of the size relationships.

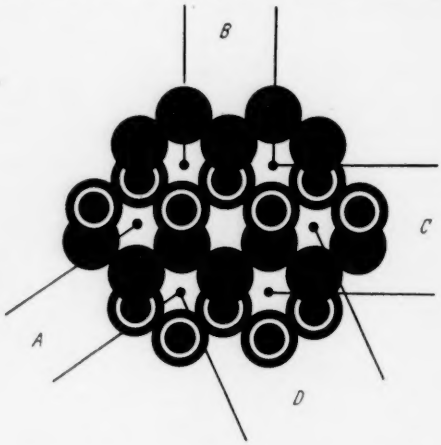

Figure 20. The crystal structure of the turnip yellow mosaic virus, in the plane of twofold symmetry. Seen from this direction the diamond lattice is perforated in this manner right through the crystal. Four layers are shown on the surface, alternately banded and solid circles from the lower edge. *A*, *B*, *C* and *D* show the directions in which measurements were taken.

of the molecules was found to be 21·8 mμ. In terms of this diameter, the lattice constants were calculated and are given in the following table. The axes mentioned are those shown in Figure 20.

Table

Axis	*Found*	*Calculated*
A	1·41	1·41
B	1·52	1·63
C	2·43	2·31
D	2·70	2·74

The size of the particle calculated from these data is of course subject to the error in microscope calibration, and must not be regarded as a measurement of high absolute accuracy.

The other possible lattice which these crystals might have had, because it was not certain that the micro-crystals had the same lattice as the large crystals from salt solutions, is the Würtzite structure, which is the hexagonal analogue of the diamond lattice. This was ruled out by the angular relationships of the axes to each other. The final structure decided upon is shown in Figure 20 in which molecules in different planes are indicated alternately as solid black and black with white rings. Each molecule is attached to four others to form a tetrahedron, suggesting that the individual molecules may also have tetrahedral symmetry.

This virus has also been examined by x-ray crystallographic techniques, both in the form of powders (BERNAL and CARLISLE[57]) and as single crystals when these became available (BERNAL and CARLISLE[58]), as well as in the form of the non-cubic crystals which this virus also forms (MARKHAM and SMITH[11]). The octahedral crystals of the virus protein and the virus nucleoprotein both have very similar structures, and differ only slightly in their cell sizes, the nucleoprotein cell being some 2 per cent smaller than that of the protein, in spite of the fact that its molecular weight is about 1 2/3 times that of the latter. The cell of the wet protein crystals is a cube with edge 71·5 mμ, and is the largest cell ever to have been measured. This cell contains eight molecules with the centres of adjacent molecules 30·8 mμ from each other, while in the case of the nucleoprotein molecules the distance between molecular centres is 30·4 mμ. The molecular diameters calculated from the diffusion constants of these two virus proteins are 28·2 mμ and 27·5 mμ respectively (MARKHAM[25]), the difference although small probably being greater than can be accounted for by experimental error, and this suggests that particles are slightly tetrahedrally deformed spheres which in the crystals are in contact through their apices, thus making their apparent diameter larger. Both types of crystal shrink on drying, and the cells also shrink, the distances between particle centres decreasing to 22·8 mμ for the nucleoprotein and 23·8 mμ for the protein components. This has been interpreted as meaning that there is a superficial layer of water as much as 7·8 mμ thick between the molecules in the wet crystals, through which the directive forces holding the molecules in the crystal lattice are acting, but the fact that it also seems to exist when the particles are in solution, as shown by the diffusion experiments, suggests that the situation is more complex and that the molecules do in fact shrink on drying. The protein component of this virus is unique in that when it is in the dry state it is almost certainly hollow, for the diameter which it has in the crystal lattice is about 1·3 times that of the equivalent solid sphere having the same molecular weight and density (3×10^6 and 1·35 g/cm^3), while the diameter of the nucleoprotein calculated as a solid sphere is 21·9 mμ. This is in reasonable agreement with the x-ray crystallographic data, particularly if one assumes, as in the case of the wet crystal, that the particles are not exactly spherical, though in this case the difference is much smaller. There is of course no *a priori* reason why protein molecules should not be hollow because there are several cases known of smaller molecules having hollow structures. The interest is merely in that it has been observed.

Other viruses

Several cases have been reported where apparent crystal formation has taken place in the case of the non-molecular viruses, but probably most

of these 'crystals' are analogous to the close packing which one observes when small spheres are thrown into a heap or when 'rafts' of bubbles are made.

THE PROPERTIES OF TOBACCO MOSAIC VIRUS SOLUTIONS

Even before the isolation of this virus it had been investigated in plant sap by TAKAHASHI and RAWLINS[36]. Tobacco mosaic virus is present in plant sap in a high concentration (1-2 mg/ml) and consequently sufficient is present to exert an appreciable influence on the latter. TAKAHASHI and RAWLINS[36] found that when infective sap was allowed to flow through a fine capillary tube, mounted on the stage of a polarising microscope with its axis at 45° to the planes of polarisation of the Nicols, the lumen of the tube became birefringent, and the birefringence was even in intensity all across the tube, while a sol containing disc-shaped particles gave rise to a stream showing birefringence only at its edges. Therefore they concluded that the virus was asymmetrical and probably rod-shaped, a conclusion which has been amply borne out by later work. It will be noted that the orientation of rods in a capillary tube will be parallel to the length of the tube right across the lumen when viewed normally, while plate-shaped particles will tend to orientate parallel to the walls of the tube, and thus will only be viewed in parallel arrays at the edges of the tube.

Most of the study of the flow birefringence of tobacco mosaic has been made by qualitative techniques rather than by the quantitative methods which were used on muscle proteins by VON MURALT and EDSALL[59], the greater number of quantitative measurements on virus solutions having been made by viscometric techniques, which give similar information with less complex equipment.

As has already been mentioned, EINSTEIN's viscosity equation is limited to dilute suspensions of spherical particles. Considerable theoretical work has been done on the analogous cases of simple asymmetrical particles, usually ellipsoids of revolution or rows of spheres. It was evident from the earliest approaches to the problem that rod-shaped particles had viscosity increments of a higher order than did spherical particles occupying the same volume, when the former were at right angles to the velocity gradients, and gradually the theory was elaborated for the case of suspensions of randomly orientated particles.

In the case of suspensions of very small rods the problem becomes rather complex unless certain simplifications are introduced. When the suspension is at rest the particles are distributed randomly both in space and in direction owing to Brownian motion. When the liquid is now set in motion, the rods tend to lose their random distribution and to occupy

a preferential orientation, which is dependent upon the rotary diffusion constant θ of the particles, and the velocity gradient β in the fluid. This preferential orientation is only statistical, that is to say it is the position in which individual rods tend to spend most of their time. In general, it is simplest to consider the cases where orientation is negligible rather than to solve the more complex problem, or, as is more usual, to ignore the orientation altogether and hope for the best. For all practical purposes it may be taken from the data of BOEDER[60] that the orientation is negligible when $\beta/\theta \ll 0{\cdot}5$ and not serious when $\beta/\theta = 1$. For a rod θ may be calculated from the formula:

$$\theta = \frac{2{\cdot}41 \times 10^{-13}}{a^3}\left(-1 + 4{\cdot}6 \log_{10} 2\frac{a}{b}\right) \quad \ldots . (9)$$

which is derived from PERRIN[61,62]. In this equation a and b are the major and minor semi-axes of the rod. For tobacco mosaic virus having $2a = 300$ mμ and $2b = 15$ mμ, θ becomes 360 sec.$^{-1}$, a dimer has a θ of about 64 sec.$^{-1}$, and a rod of 1200 mμ length one of about 8·8 sec.$^{-1}$. (These figures neglect the effects of solvation of the particles which would probably have the effect of decreasing θ slightly). For a normal precision viscometer of the capillary type, $\bar{\beta}$ (the mean value of β) may be calculated from:

$$\bar{\beta} = 0{\cdot}85\, v/r^3 \quad \ldots . (10)$$

and

$$\beta_{max} = 1{\cdot}27\, v/r^3 \quad \ldots . (11)$$

where β_{max} is the gradient at the walls of the tube of radius r, through which a volume of fluid v is flowing every second, and this is of the order of 900 sec.$^{-1}$. (These formulae are derived from KROEPELIN[63]). It will be seen that such viscometers are unlikely to give results of much absolute accuracy for normal tobacco mosaic virus preparations. LAUFFER[64] has used an instrument with a $\bar{\beta}$ of 350 sec.$^{-1}$, and it is unlikely that his results will be in much error, especially as a finite time is required for the steady state to be set up, so that as the liquid is flowing through the tube rapidly, the effect of orientation in capillary viscometers may be rather less than one would expect for the steady state condition. The results are usually expressed by means of an equation derived by SIMHA[65]:

$$\eta_{sp}/\phi = \mathcal{J}^2/15\,(\log 2\mathcal{J} - 3/2) + \mathcal{J}^2/5\,(\log 2\mathcal{J} - \tfrac{1}{2}) + 14/15 \quad \ldots . (12)$$

where $\mathcal{J}$ is a/b and ϕ is the *volume* fraction of material in solution, and $\eta_{sp} = \eta_{rel} - 1$. ϕ is however usually taken as being the dry volume of the material in solution. MARKHAM *et al.*[6] have pointed out that, provided solvation increases the width of particles rather than their length, the value of a/b deduced in this way from viscosity measurements is a close approximation to the axial ratio of the dry particles. There is however no *a priori*

reason for expecting solvation to increase two of the dimensions and not the third. Values of axial ratios should therefore be accepted with a little reserve, unless there is some information about the extent of the solvation, if any. Furthermore, as will be seen from SIMHA's equation, if the suspension is not monodisperse, the average value for the axial ratio given will be weighted towards that of the longer particles, though in capillary viscometers this factor will be minimised by the increased degree of orientation of the latter.

FRAMPTON[66] has designed a capillary viscometer of a type where $\bar{\beta}$ varies from a maximum to zero during a single run, it being possible to make measurements in this apparatus from values of $\bar{\beta}$ from (calculated from FRAMPTON's data) 60 sec.$^{-1}$ to as low as 10 sec.$^{-1}$. Unfortunately it is a matter of great difficulty to calculate the viscosity of non-ideal solutions from the experimental data obtained in this kind of apparatus, and it may be pointed out here that FRAMPTON's own results are calculated by assuming tacitly that the value of $\eta_{rel}-1\ /\phi$ is not varying with $\bar{\beta}$, whereas the same results are used later to show that it is. Using a greatly aggregated specimen FRAMPTON found that, as the velocity gradient in his viscometer decreased, η_{sp}/ϕ increased to a value of just over 400, and was still rising. Misled by the shape of his curve of η_{sp}/ϕ against the pressure head, he concluded that at zero flow rate the viscosity of his solution would be infinite, or in other words, that the suspension was a thixotropic gel. At about the same time ROBINSON[67] published a detailed study of the viscosity and flow birefringence of a single specimen of aggregated tobacco mosaic virus, using a coaxial cylinder apparatus. In this type of apparatus

$$\beta = \frac{R\omega}{d} \qquad \ldots\ldots (13)$$

where R is the average radius of the two cylinders, and d is the width of the annular space between them, ω being the angular velocity of the external cylinder. (This formula only applies for cylinders with radii large compared with d.) It will be noted that very small values of β can be obtained with such an apparatus, and ROBINSON made measurements at as low values of β as 0·36 sec^{-1}, when orientation was apparently negligible. As was to be expected η_{sp}/ϕ rose to a steady value, in this case to about 1450, corresponding to a particle length calculated from SIMHA's equation of some 2·5 μ. ROBINSON was also able by means of his apparatus to measure θ and the amount of birefringence of his virus solution and thus to correct approximately for the orientation of his viscometer. His formula relating η_{sp}/ϕ and a/b gives values for the latter about half that given by SIMHA's equation.

As has been indicated earlier, these and many other similar measurements have been made on aggregated virus material, which was certainly

both polydisperse and much longer than the state in which it existed originally in the plant, while the particle lengths were so long that flow through a capillary tube would have been sufficient to break them down into shorter pieces. In fact with aggregated virus it is almost impossible to transfer the solutions without causing a detectable change in viscosity because of this factor. The solutions which LAUFFER used were probably little aggregated and his value for η_{sp}/ϕ of 39·0 is the best one obtained so far[64]. This result gives a value of a/b of about 20 which, although subject to error from hydration effects, is in good agreement with the data obtained by other methods.

Flow birefringence measurements may be used to give a direct measure of θ, but the theory of such techniques is complex and the results for mixtures are very heavily weighted towards the longest particles. Qualitative measurements of flow birefringence are therefore exceedingly sensitive indications of change in particle lengths and it is possible by this technique to demonstrate the decrease in particle length resulting from the shaking of a test tube full of aggregated virus.

The effect on such solutions of more violent disruptive forces has been studied by OSTER[68] who investigated the effect of ultrasonic vibrations on solutions of the virus. He found that the viscosity of the solutions fell off rapidly with ultrasonic irradiation, and that this was correlated both with an increase in the number of small fragments seen under the electron microscope, and also with a decrease in infectivity. These observations are of interest because they cast doubts upon the significance of the small fragments which are liberated from diseased plants by drastic milling processes (BAWDEN and PIRIE[69]) and which, though related immunologically to the virus, are non-infectious.

Solutions of this virus have also been used in the study of the light-scattering behaviour of suspensions of rods by OSTER, DOTY and ZIMM[70]. In the case of elongated molecules or particles of the order of size of the wavelength of the light used, light scattered in a forward direction is more intense than the light scattered at an equal angle to the beam in a backward direction, and the longest dimension of a rod-shaped particle may be determined by the measurement of this ratio. These authors used a centrifugally prepared specimen of the virus and calibrated it by electron microscopical observation and showed that 67 per cent of the rods had a length of 280 $\pm$ 20 mμ. Viscosity measurements made with an OSTWALD viscometer gave a value of a/b of 17·3, calculated from SIMHA's equation, or a length of about 260 mμ, which is probably an underestimate for reasons which have already been mentioned.

The light scattered from solutions of this material at two angles symmetrical to the incident beam (42·5° and 137·5° in air) were measured, and from the intensity ratio (dissymmetry) extrapolated to zero concen-

tration, they calculated the particle length in terms of the wavelength of the light used. This came out as 270 mμ. In stronger solutions, particularly in the absence of salt, the rods were not independent of each other, as is demanded for the application of the theory of light scatter to this problem. Correcting for the dissymmetry in the light scattering these authors also calculated the dry molecular weight of the particles and found a weight average of $M_0 = 40 \times 10^6$. This value is in excellent agreement with those obtained by other methods, the best of which is probably that obtained by WILLIAMS, BACKUS and STEERE[71] who by a direct weighing method obtained a value of 49×10^6 on a preparation having a particle length of 298 ± 1 mμ (WILLIAMS and STEERE[72]). In this work corrections were necessary for the electron microscope abberations. It is of course a matter of extreme difficulty to measure the molecular weight of any asymmetrical particle with certainty, and the results on the tobacco mosaic virus demonstrate how reliable many of the physico-chemical methods can be on uniform or nearly uniform suspensions.

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5

MECHANISMS OF BIOLOGICAL ACTION OF ULTRAVIOLET AND VISIBLE RADIATIONS

Maurice Errera

In studying the biological effects of U.V. or visible light, three major questions arise : (*a*) what is the mechanism of energy transfer to the cell substrates; (*b*) which of these components are damaged and (*c*) how do these transformations bring about biological effects. In the first part of this paper, we would like to discuss some considerations on the possible mechanisms of photobiological effects : both direct and indirect effects seem plausible from results of model experiments. In the second part, we would like to find out which cellular constituents undergo some sort of damage in the course of irradiation and how permanent is this damage. We will conclude by discussing how these effects are liable to alter the behaviour of the irradiated organisms.

MECHANISMS OF ACTION OF U.V. AND VISIBLE LIGHT ON ISOLATED SUBSTRATES

DIRECT PHOTOCHEMICAL EFFECTS ON BIOLOGICAL SUBSTRATES

We shall not attempt to make a complete survey of the photochemical properties of biological compounds, although a complete knowledge of these is necessary for the full understanding of biological effects of radiations. This survey has been done for proteins[176] and for proteins and nucleic acids[93,94,166,73], so we think it preferable to see how data from more recent papers fit into the general picture which these reviews suggested.

Irradiation of proteins, or related molecules, may result in the breakage of simple bonds, such as hydrogen bonds, peptide and amide bonds, disulphide bonds, provided the wavelength used is absorbed (< 300 mμ); deamination and decarboxylation occur frequently; simple aldehydes and ketones may be decomposed[171]; the hydrogen atoms of the aromatic ring of amino-acids may be oxidised to the —OH group. However when proteins are irradiated one does not find the expected increase in the number of free amino and carboxyl groups, as would be expected from the breakage of peptide bonds observed in simple peptides or hemi-

peptides (McLEAN and GIESE[178]). And when looked for, low molecular weight peptides and amino acids are frequently not found after protein irradiation, as is the case for lyzozyme[212]; when insulin is inactivated, liberation of tyrosine is not sufficient to account for loss of biological activity[137]. If one supposes that peptide bonds are split during irradiation, aggregation of low molecular weight compounds may explain these facts : when bonds are opened, the radicals formed will have a tendency to diffuse away from each other if the fragments are small as in peptides, but if the fragments are large as in proteins and viruses, high diffusion coefficients and cross linkages keep them together, thus increasing the probability of recombination (FRANK-RABINOVITCH effect). Ultraviolet light is known to be capable of inducing condensation of peptides as in the case of diketopiperazine to glycyl glycine[135]. This may be the reason why the quantum yields for photolysis of high molecular compounds like proteins, nucleic acids or viruses are small (10^{-2} to 10^{-5}) and it may be one explanation of the finding of McLAREN, that a roughly linear relation between $1/\emptyset$ ($\emptyset$=quantum yield) and the log of the molecular weight is obtained. Although, as McLAREN remarks[166] no comparisons are available for identical substances at the same wavelength, it appears from MITCHELL and RIDEAL'S work[184a], that the quantum yield for photochemical damage of a protein molecule is higher when this molecule is unfolded at a water-air interface : in this case, a primary step for denaturation is provided by the unfolding of the molecule and the photolysed fragments have the possibility of diffusing into the aqueous medium where the chances for recombination will be small.

It is important for the full understanding of photochemical properties of macromolecules to investigate *all* possible alterations, which are not necessarily simultaneous or related to biological inactivation.

SHUGAR, for instance, finds lyzozyme to be inactivated at 253·7 mμ with a quantum yield of 0·024, but increase in U.V. absorption at 280 mμ only begins when a considerable part of the enzyme is already inactivated[212]. Two major changes occur during irradiation of serum albumin, the first being a change involving tyrosine groups, the second a photo-oxidative step leading to denaturation (KOFMAN[149]). Another instance is provided by polymerised desoxyribonucleic acid: if degradation is assayed by viscosity measurements and if one postulates that loss of asymmetry of each particle is the result of the absorption of a single quantum, a very low quantum yield is found ($< 10^{-5}$)[70]. This change is however accompanied by destruction of pyrimidine rings, loss of affinity for methyl green, slight dephosphorylation and deamination : furthermore molecular weight does not drop considerably (ELY and ROSS[65]) and very little dialysable material appears. All these changes require energy, but it is not known to what extent alteration of these chemical properties influences

the viscosity of the solution. If one compares[70] the quantum yield for photolysis of pyrimidines in the high molecular desoxyribonucleic acid or in the low molecular prosthetic group of thiamine (UBER and VERBRUGGE[243]), they appear to be of the same order of magnitude (about 5 times less in the macromolecule, but this may be due to experimental inaccuracy).

Quantum yields are often dependent on the conditions chosen for irradiation : they may be lower near the isoelectric point as in chymotrypsin (FINKELSTEIN and MCLAREN[80]), in high salt or enzyme concentrations, as in triosephosphate dehydrogenase (RAPKINE[196]; SHUGAR[214]). These effects may be due to an increase in recombination of photolysed fragments, but are far from general : photolysis of lyzozyme proceeds at the same rate over a wide range of *p*H (from *p*H 3·0 to 12·0), the isoelectric point being near *p*H 10·5 (SHUGAR[212]). Aldolase has the same sensitivity over a 24 fold concentration range[152b]; the same is probably true for lyzozyme[212] and desoxyribonucleic acid[70].

More subtle photochemical changes have also been described in the case of simple molecules where analytical determinations are easier, like trans-cis transformations of azobenzene (HARTLEY[107]). Similar minute effects may be significant in determining biological inactivation.

Attempts to find the relation between alteration of chemical structure of irradiated molecules and loss of biological activity have so far been lacking. Essential chemical groups necessary for enzymatic activity are now beginning to be known in a small number of cases; a quantitative analysis of the amino acids and peptides obtained by gradual hydrolysis, of irradiated proteins might be able to help discover if the loss of biological activity is due to alteration of specific groups or only to rearrangements of these within the molecule.

The case of glutamic and aspartic transaminase is interesting in this respect : the system is rather radio-resistant to ionising radiations, and its sensitivity to U.V. is due in all probability to photo-destruction of pyridoxine, which probably is an essential prosthetic group of this system (SCHLENK[205]).

The following interpretation of photochemical processes concerning proteins had been given by CLARK in 1939; in its main thesis it still is compatible with most of the recent results. A first step, purely photochemical, is the excitation process leading to breakage of chemical bonds, to dissipation of energy to a neighbouring molecule or molecular group within the absorbing molecule (photosensitisation or 'intramolecular' photosensitisation) or to the medium in which case the energy is lost as heat. If fragments are formed, they may be kept together through H bonds between lateral chains, but are also liable to yield to secondary conditions such as a rise in temperature. This secondary step may be accompanied

by intramolecular rearrangements and may possibly be influenced by light although it is essentially a dark reaction; finally aggregation occurs.

In most instances so far studied, biological activity appears to be lost during the first photochemical step : when tobacco mosaic particles are inactivated, no chemical, serological or physical changes can be detected : crystallisation of the virus is still possible, absorption spectrum and viscosity of the solutions are not changed[189]; one can however detect an increased instability when the temperature is raised, which is a frequent finding : RAPKINE has also observed it with triose phosphate dehydrogenase[196]. The same thing occurs in ovalbumin solutions and in this case there is a concomitant rise in absorption at 280 mμ; dodecyl sulfate can prevent this aggregation.

Sulfhydryl groups—The photochemical reactions of SH groups are important : their oxidation by X-rays has been postulated by BARRON[10] to be an important step in biological actions of ionising radiations. As most fundamental effects of ionising radiations can also be induced by U.V. (inhibition of mitosis, killing of cells, mutations) one expects to find that similar constituents are finally involved in both cases. SH groups are oxidised by ionising radiation, and the reduction of S-S linkages is negligible as in the case of glutathione[10] and triose phosphate dehydrogenase[215]. The situation is not as clear when one considers U.V. radiations. Cysteine, reduced thioglycolic acid or glutathione are oxidised to the —S-S— compounds; in the latter case a low quantum yield is found, which has been interpreted to indicate a reversible reaction[64].

In addition, numerous examples of the appearance of SH groups in irradiated proteins indicate that these are unmasked as in heat or other denaturation processes (many references in [176]). During ultraviolet denaturation of triose phosphate dehydrogenase, oxydation of SH groups does not appear to be a major step[212], as is the case for inactivation of SH enzymes by ionising radiations[10]. Urease, another SH enzyme, is not reactivated by reducing agents or tissue extracts (PILLEMER[191]); however TAUBER found it could be reactivated by a boiled urease preparation[235].

POSSIBLE INDIRECT EFFECTS

In recent years, the possibility of indirect biological effects of ionising radiations has been considerably emphasised owing chiefly to the work of FRICKE[82], DALE[54] and LEA[163]. These indirect effects are due to the decomposition of water molecules into short lived active free radicals or atoms ($OH^{\bullet}$, $H^{\bullet}$, O_2H . . .) and the production of more stable peroxides (H_2O_2, organic peroxides)*. The possibility of producing these same agents by the action of U.V. light must not be neglected in trying to understand the mechanism of biological effects of these radiations.

* For references, see the recent Discussion of the Faraday Society (April 1952).

Water does not absorb U.V. light above 1850 mμ, so activation of water will only occur in the presence of sensitising molecules. At 253,7 mμ absorption of light by oxygen in solution seems almost negligible; but ozone, often formed in the vicinity of Hg arcs, may diffuse into the reaction medium and this complication has to be avoided[64]. We have summarised some of the data in which the occurrence of $H^{\bullet}$, $OH^{\bullet}$, H^2O^2 or peroxides seem to be formed under the influence of U.V. radiations. The following schemes have been proposed to explain some of the reactions.

(*a*) In the case of *ferric ions*. (EVANS and URI[74,74a])

1. $Fe^{3+}OH^{-} \overset{h\nu}{\rightleftharpoons} Fe^{2+}OH$ excitation and primary dark back reaction
2. $Fe^{2+}OH \longrightarrow Fe^{2+}+OH^{\bullet}$
3. $Fe^{2+}+OH^{\bullet} \longrightarrow Fe^{3+}+OH^{-}$ secondary dark back reaction
4. $Fe^{2+}OH+M \longrightarrow HOM+Fe^{2+}$
   $OH^{\bullet}+M \longrightarrow HOM$
5. $OH^{\bullet}$ recombination or subsequent reaction

(*b*) During irradiation of I^{-}, SH^{-}, RS^{-}, Fe^{2+}, Ce^{4+} irradiation also leads to the production of $OH^{\bullet}$ [74a,254b], H atoms are probably liberated which in acidic condition give rise to Hydrogen (H_2^{+}) molecules, these being electron acceptors may act as oxidant (WEISS [254a,254b]).

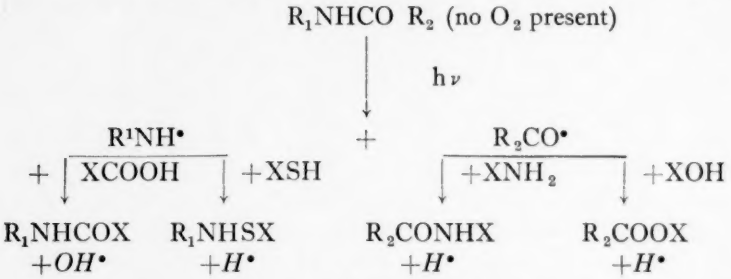
(*c*) U.V. irradiation of ascorbic acid leads to oxidation and H_2O_2 is accumulated if oxygen is present; in its absence only traces of H_2O_2 can be detected, but hydrogen is evolved (GUILLEMET[101]).

(*d*) In the case of gazeous *acetic acid* (see WATERS[278]).

1. $CH_3COOH \overset{h\nu}{\longrightarrow} CH_3COO^{\bullet}+H^{\bullet}$
2. $CH_3COOH+H^{\bullet} \longrightarrow H_2+CH_3COO^{\bullet}$
3. $CH_3COO^{\bullet}+H^{\bullet} \longrightarrow CH_4+{}^{\bullet}COOH$
4. ${}^{\bullet}COOH \longrightarrow CO+OH^{\bullet}$
5. $2CH_3COO^{\bullet} \quad C_2H_6+2CO_2$

Acetic acid does not absorb U.V. at wavelengths higher than 240 mμ; but similar reactions of acids containing chromophore groups would of course be interesting to investigate.

(*e*) In the case of *proteins*, ROBERTS[198] suggests the following schemes.



In the presence of O_2 more $OH^{\bullet}$ might be formed, which would react with $R_1NH^{\bullet}$ and $R_2CO^{\bullet}$ to form R_1NHOH and R_2COOH ; furthermore ozone becomes detectable. ROBERTS explains that in O_2, the secondary decrease in aggregation which is observed is due to the direct combination of radicals formed by the splitting of the peptide bond with $OH^{\bullet}$, thus preventing their combinations to form heavier aggregates. Some evidence for the preceding scheme comes from the polymerisation of acrylonitrile if this compound is added to the protein solution when irradiated in N_2. Furthermore, if irradiation of the protein proceeds in the presence of low molecular weight compounds containing carboxyl, amino or hydroxyl groups (acetic, succinic or citric acid; methylamine, ethylenediamine, tyrosine or quinol) the aggregation is inhibited because the peptide radicals formed during irradiation combine with the low molecular weight compounds which act competitively. When irradiated in O_2, the protein releases low molecular tyrosine and tryptophane containing molecules which do not occur in the absence of O_2. These results appear to be contradictory to those of GIESE and MCLEAN and of SHUGAR (p. 89) and should be investigated with other proteins. FIALA has demonstrated polarographically, a non-oxidative denaturation of ovalbumin; in the presence of oxygen, a photooxidation follows this primary step; the mechanisms of both steps are still obscure, but oxygen might be taken up by the protein or act as a dehydrogenation agent with the formation of H_2O_2 [78].

Let us remark that the splitting of the peptide bond, whose absorption is very low in the 260-280 mμ region, would chiefly be due to intramolecular sensitisation (BEAVEN, HOLIDAY and JOPE[14]).

(*f*) In addition to $H^{\bullet}$, $OH^{\bullet}$ and H_2O_2 formation during irradiation of organic molecules, a certain number of peroxides have been shown to arise (for references see SCHÖNBERG[207]). Amongst organic compounds related to substances of biological interest, which are liable to be oxidised to organic peroxides are derivatives of benzene; acetic acid and benzaldehyde give peracetic and perbenzoic acid; and numerous cyclic

compounds can be oxidised to straight or transannular peroxides an example of which is ergosterol peroxide obtained by irradiating ergosterol in visible light using eosine as sensitiser.

RÔLE OF OXYGEN

It appears from what precedes[78,198] that the presence of oxygen is not necessary for photolytic activity of U.V. light on amino acids or proteins, although it does influence to a considerable extent the appearance of secondary reactions. MANDL, LEVY and MCLAREN[171] have observed that both U.V. deamination of peptide derivatives as well as increase in U.V. absorption is more effective when the substrate is irradiated in the presence of O_2; oxygen seems essential for U.V. oxidation of tyrosine (ARNOW[7]). Other important photo-oxidative reactions concerning amino acids have been reviewed by MCLAREN[176]. When proteins are irradiated in the presence of oxygen, we have seen that, as ROBERTS reported, photochemical reactions are different. BECKER and SZENDRO had already seen in 1931 that irradiating ovalbumin or serum proteins in O_2 or N_2 gave different results: in O_2 the aromatic amino acid absorption peak near 280 mμ is wiped out, whereas it is still present after irradiation in N_2[15].

When enzymes[80] or virus[189] solutions are irradiated in N_2 or O_2, the quantum yield for inactivation is identical, which indicates that loss of biological activity is due to the primary effect and not to secondary oxidative steps. This is in line with the view advanced previously that changes in U.V. absorption and loss of biological activity are not necessarily related as in the case in lyzozyme[212], insulin[137] and chymotrypsin[80] inactivation.

After effects—The occurrence of after-effects such as change in absorption spectrum and aggregation are frequent; they are often better demonstrated when irradiation takes place at low temperatures and that the solution is heated afterwards (CLARK[46]). Even when irradiation takes place in N_2, in the presence of luminol, a definite chemiliminescence is visible still 30 minutes after irradiation has stopped, suggesting a possible retarded action of H_2O_2[198]. SPIEGEL ADOLF also observed late changes in absorption spectra after irradiation of proteins in nitrogen or oxygen[217] and bleaching of methylene blue continues for some time after U.V. irradiation[190a].

RÔLE OF PROTECTING AGENTS

The rôle of protecting agents is not as clear in the case of U.V. as it is for ionising radiations; many cases seem to need independent consideration.

(*a*) When oxidations are involved as in the case of aromatic acids, there is a definite effect of sulfur containing amino acids : benzoic acid, phenylalanine, tyrosine, histidine are less easily photo-oxidised in the presence of cysteine, cystine, homocystine or methionine (SCHOKEN[206]).

(*b*) Photo-oxidation by visible or U.V. light of pteroylglutamic acid, a reaction which is slowed down in the absence of oxygen, is considerably inhibited in the presence of p. amino benzoic acid[195]. No explanation for this effect has been advanced, but it is possible that the fluorescent substrate sensitises some alteration of the protecting agent, in a manner similar to the effect of tobacco mosaic virus on acriflavine : the bleaching of the dye is much faster in the absence of the virus (OSTER and MCLAREN[189]).

(*c*) We have already mentioned the protecting effect of carboxylic or hydroxyl compounds, which inhibit aggregation of serum albumin, irradiated in concentrated solutions (1 per cent). This is probably not a competition for OH radicals, but perhaps for peptide radicals because thiol groups (which are excellent protectors in the case of ionising radiations) and amino groups do not give any protection in this case[198]; let us note here that with these protein concentrations, one does not observe much protection effect, of tobacco mosaic virus[165] or fowl erythrocyte nucleoprotein[69] submitted to ionising radiations.

(*d*) Yet another protective effect is that of cysteine on triose phosphate dehydrogenase (SHUGAR[214]). If a partly oxidised preparation of the enzyme is reduced prior to irradiation, the enzyme becomes more resistant. A completely oxidised sample, which must be reduced by cysteine prior to assay, is inactivated more rapidly still. SHUGAR suggests that the presence of —S-S— linkages may alter the configuration of the protein and possibly enhance the transmission of absorbed energy from the chromophores to other parts of the molecule and thus increase the rate of inactivation.

(*e*) Lyzozyme is not protected by cysteine, its S-S groups may be less readily reduced; it is to a slight extent by arginine which was tried in view of the high content of lyzozyme in guanidyl groups[212].

(*f*) Secondary reactions may also be prevented by different agents : caprylate added after irradiation of serum albumin is able to minimise both the increase in viscosity of the solution in the presence of dodecylsulphate and the rise in absorption[61]. The mechanism of this effect is still obscure.

As a result of these findings, no general theory of protection of proteins during photo-inactivation can be logically formulated. In the case of ionising radiations, competition of the protecting agent for radicals

produced by ionisation of water, is a general phenomenon; in the case of ultraviolet radiations, the protecting action seems to depend much more on the nature of the photochemical process and on the nature of the organic radicals released.

PHOTOSENSITISED REACTIONS

BLUM[18] has already given some of the general characteristics of photosensitised oxidations. Although H_2O_2 has been demonstrated during irradiation of fluorescein dyes, the presence of hydrogen peroxide does not seem essential for effects obtained by illumination with visible light of various biological systems. As many substrates are chemically altered through these photosensitised reactions, it seems worth while to review some of the recent data. Many references concerning light absorption by dyes and photosensitisation may be found in a recent review by BOWEN[25].

(*a*) *Amino acids*

Amino acids irradiated (·0025 m moles/ml) in the presence of methylene blue (MB) has led WEIL and his collaborators to the following conclusions.

1. *Methionine* is dehydrogenated and leads to the formation of methionine sulphoxide with the formation of H^2O^2; the following scheme is suggested[250].

$$\text{Methione} + \text{MB} \xrightarrow{h\nu} \text{dehydrogenated methionine} + \text{MB}H_2$$
$$\text{MB}H_2 + O_2 \longrightarrow \text{MB} + H^2O^2$$
$$\text{Dehydrog. Meth.} + H^2O \longrightarrow \text{Methionine sulphoxide}$$

If irradiation takes place in N_2, methylene blue is bleached which is similar to what happens when nicotine[252] is irradiated, in the presence of the dye; H_2O_2 formation is a secondary reaction. Cysteine, in the same conditions, is oxidised to cysteic acid and perhaps further.

2. *Aromatic amino acids. Tyrosine* oxidation is accompanied by a decrease of specific U.V. absorption indicating a photolytic process involving the aromatic ring; no melanin is formed; phenylalanine in the same conditions appears to be unchanged. In *tryptophane*, oxidation of the pyrrole ring occurs probably faster than that of the benzene ring. *Histidine* or *Histamine* irradiation leads to the opening of the imidazole ring with probable liberation of aldehydic groups. Although no evidence for H_2O_2 formation has been found, formation of organic peroxides, not susceptible to the action of catalase, during tryptophane and tyrosine irradiation is probable. Riboflavin[86,87] or other fluorescing dyes (eosin *etc.*[75]) have been used to study the photo-oxidation of indoleacetic acid (IAA) and other indole derivatives which are denatured and also give

condensation products[87]; when oxygen is present H_2O_2 is evolved. This mechanism of photosensitised destruction of indole acetic acid, a plant hormone, is suggested as the natural one involved in phototropism : light destroys the hormone on the most illuminated side of the plant, thus giving rise to the maximum growth on the opposite side.

In the absence of air, riboflavin irradiated in solution with indole acetic acid is bleached, and hardly oxidised. If O_2 is introduced into the vessel, disappearance of indoleacetic acid is very rapid.

$$\begin{aligned} & \xrightarrow{h\nu} \\ \text{Rbf} & \longrightarrow \text{Rbf}^* \\ \text{IAA} + \text{Rbf}^* & \longrightarrow \text{oxidation product} + \text{Rbf H}_2 \\ \text{Rbf H}^2 + 1/2\ \text{O}^2 & \longrightarrow \text{H}^2\text{O} \end{aligned}$$

Activated riboflavin (Rbf*) thus acts as a hydrogen carrier between indole acetic acid and oxygen; CO_2 is evolved during indole acetic acid photolysis in almost equimolar quantities to O_2 uptake[87]. It may be interesting to note that the quantum yield for the inactivation has been measured by GALSTON : using blue light (430-550 mμ) it is of the order of 0·6; SHUGAR found a very similar value using near U.V. light (360 mμ[210]); in the same conditions, tryptophane is photo-oxidised with a lower quantum yield ($3{\cdot}8\ 10^{-2}$). As we shall see, the indole ring may undergo similar changes when incorporated into a protein molecule.

3. *Other amino acids* generally do not take up much oxygen on illumination in the presence of dyes (methylene blue[250]), and the presence of a peptide bond does not increase oxygen uptake. This of course is in contrast to what happens during direct ultraviolet irradiation where peptide bond splitting appears to be one of the major photochemical reactions.

(*b*) *Proteins*

1. GAFFRON[84] has irradiated and oxidised serum proteins in the presence of dyes (hematoporphyrin, chlorophyll): CO_2 and NH_3 were liberated. Numerous other examples have been reviewed by HEYROTH[64] and BLUM[18]. More recent experiments on crystalline enzymes have shown essentially the same thing : if lyzozyme is irradiated with visible light in the presence of methylene blue, oxygen is taken up by the solution, tryptophane is oxidised and loss of enzymatic activity occurs; this suggests the importance of tryptophane for this activity; tyrosine seems to be oxidised in a later step[251]. SHUGAR compared the behaviour of crystalline lyzozyme and ribonuclease (this last enzyme contains no tryptophane) when irradiated with visible light in the presence of riboflavin. The fact that lyzozyme alone ($\emptyset = 0{\cdot}006$ at 253·7 mμ and 360 mμ) is inactivated, again emphasises the importance of tryptophane for photosensitised inactivation.

That tryptophane is altered is proved by the disappearance of the glyoxylic reaction of BRICE. The quantum yield for the oxidation of tryptophane, both free and included in the protein molecule, is of the same order of magnitude ($\sim 0{\cdot}04$)[210]. Riboflavin has also been used by GALSTON and BAKER[88] to photosensitise the inactivation of a series of enzymes (α amylase, urease, tyrosinase).

A very recent report of WEIL and BUCHERT indicates that in the presence of methylene blue the photo-oxidation of the histidine and tryptophane of β lactoglobulin is very rapid and occurs for the same oxygen uptake than in the case of the isolated amino acids; the oxidation of cysteine is slower and that of tyrosine only begins when the two first amino acids are completely oxidised; methionine is not affected at all, although it is considerably when isolated. These changes are accompanied by a marked shift towards the shorter wavelength of the original absorption maximum, this is accompanied by a general rise in absorption over the entire U.V. range; there is also a considerable rise in molecular weight and an increase in insolubility at the isoelectric point. No extra free amino groups are detectable at the end of the experiment, indicating that peptide bonds remain perhaps intact. Gelatin and salmine, which contain little or no histidine, tryptophane, tyrosine or cystine take up only minute amounts of oxygen; lyzozyme and insulin on the contrary have an O_2 uptake even greater than β lactoglobulin[252a].

Invertase has also been inactivated in the presence of riboflavin after purification, but not when spores of *Myrothecium verrucaria* were irradiated; even after homogeneisation of the spores no inactivation occurs[169].

As FERRI has shown, indole acetic acid may be inactivated by other fluorescent dyes than riboflavin. Snake poisons have also been inactivated by photo-oxidation in presence of fluorescent dyes including chlorophyll[75,76,77]. Crystalline oestrogens undergo marked changes in U.V. absorption spectrum and loss of biological activity if exposed to visible light in the presence of riboflavin or lumichrome; $MnCl_2$ has a marked protecting activity[187].

2. Polarographic observations have been made by FIALA[79] during irradiation of serum proteins in the presence of dyes of the fluorescein serie. Although irradiated alone, these dyes produce H_2O_2 which is accompanied by O_2 uptake, this uptake is considerably increased in the presence of protein, but no H_2O_2 is formed. Alteration of protein structure is accompanied by the appearance of free SH groups as in other denaturation processes (including U.V.) and an increase in absorption of aromatic amino acids at 280 mμ can be detected. Protein is bound to the dye in the solution, its absorption maximum being shifted; however the mechanism of oxidation is not yet clear and no further attempt has so far been made to characterise oxidation products.

3. *Photosensitised reduction of —S-S— groups*—SHUGAR[214] has recently been able to photo-reactivate an SH enzyme, triosephosphate dehydrogenase whose sulfhydryl groups had previously been spontaneously oxidised on standing. This reactivation was possible by irradiating with near ultraviolet light a solution of the oxidised enzyme in the presence of its coenzyme, diphosphopyridine nucleotide, which is apparently bound in the neighbourhood of the enzyme SH. Energy is here absorbed solely by the pyridine group of the reduced coenzyme and during the irradiation its characteristic absorption at 340 mμ is not decreased and it can still be oxidised by the sodium pyruvate-lactic dehydrogenase system. Such an effect is not obtained with x-rays; this agrees well with the findings of BARRON on the inefficiency of ionising radiation for —S-S— group reduction.

It may be mentioned here that irradiation of *Paramecia* by near U.V. ($>$ 330 mμ) induces the appearance of SH groups (CALCUTT[38]).

4. Very curious results, yet difficult to interpret on a photochemical basis, have been obtained on muscle proteins by Soviet investigators. In the presence of methylene blue (10^{-4}M) illuminated *myosin* solution show a strong increase in viscosity; however if adenosine triphosphate is now added and illumination continued, the viscosity falls (ENGELHARDT[67]). In the presence of erythrosin, myosin solution become gels on illumination; the same occurs with actomyosin in which case the gel is thixotropic[204]. The authors suggest the participation of SH groups in gel formation which is in line with FIALA's finding[79].

(*c*) *Nucleic acids*

Methylene blue or eosin photosensitise desoxyribonucleic solutions the viscosity of which becomes reduced on illumination; the same effect is obtained by irradiation with near ultraviolet light in the presence of 1·2 benzanthracene or 20 methylcholanthrene[148]. This may have some bearing on the photosensitised killing or mutation induction with the aid of similar dyes[120].

OSTER and MCLAREN have made a more detailed study of photosensitised inactivation of tobacco mosaic virus, a ribose nucleoprotein[189]. Acriflavin, a basic dye which combines with virus particles under suitable *p*H conditions, was used: irradiation with visible light induces a loss of infective activity. Acriflavin if irradiated alone is bleached; it is partly protected by the presence of the virus. Although inactivation occurs also in the absence of oxygen, it is much faster if O_2 is bubbled through the solution during irradiation. The presence of salt which probably dissociates the dye-virus complex, reduces the rate of virus inactivation.

T_{2r} virus has also been inactivated in the presence of riboflavin by GALSTON and BAKER[88].

(d) Photosynthesis

One of the most important biochemical photosensitised reactions involving some kind of 'splitting' of the water molecule is undoubtedly that involved in the primary step of photosynthesis. We believe some aspects of these studies are worth mentioning here on account of their importance as fundamental reactions and because the methods used might very usefully be applied in other photobiological work.

In order to analyse the mechanism of these reactions, experiments were devised where illuminated chloroplasts are shown to liberate oxygen from the medium in the presence of different reducible agents. Since HILL's classical experiments[115,116], many substances have been reduced in these conditions (ferric ions, many reducible dyes). (Reviews in [117,125,126]). These reducing reactions accompanied by O_2 evolution have been followed by manometric or potentiometric oxido-reduction determinations[220]. CO_2 is the natural substrate which is reduced, it thus becomes organically bound (RUBEN[203]). More recently, search for natural H acceptors in the HILL reaction has continued and cytochrome C[179], methemoglobin[117] and oxygen[180] have been found active.

Very significant results were obtained by VISHNIAK and OCHOA[245], and by TOLMACH and GAFFRON[240,241]. These investigators have demonstrated that di- or triphosphopyridine nucleotide, important coenzymes effective in numerous biological dehydrogenations, are reduced in the presence of illuminated chloroplasts. This photochemical system was successfully linked with the pyruvate-lactate reaction when washed grana were incubated with crystalline lactic dehydrogenase preparations catalysed by $DPNH_2$ (the reduced form of diphosphopyridine nucleotide) and with OCHOA's malic enzyme, highly purified from pigeon liver and activated by Mn^{++} and $TPNH_2$ (reduced form of triphosphopyridine nucleotide). These reduced coenzymes are necessary H donors for these reductions and they are continuously regenerated by the HILL reaction. The malate reaction is a possible mechanism by which CO_2 can be bound in organic form and enter the tricarboxylic acid cycle which is now known to be involved in the edification of high energy phosphate bonds. In support of this possibility, phosphate probably does become esterified during photosynthesis in Chlorella (KANDLER[136]).

This very brief description gives an idea of a mechanism by which photochemical effects may influence biochemical reactions by altering the state of coenzymes and also a possible mechanism for storage of light energy.

A second important point may be raised during the study of chloroplasts. Although H_2O_2 can accumulate in isolated chloroplast systems from the reduction of atmospheric oxygen, this reaction does not seem to be of fundamental importance in photosynthesis. In the presence of catalase,

photosynthesis is not inhibited (GAFFRON[83]) and MEHLER has observed that evolution of O_2 by illuminated chloroplasts does not involve the intermediate production of H_2O_2 in a form available for catalase, but the formation of other peroxides is very probable[91,150,181,182].

ATTEMPTS TO IDENTIFY SENSITIVE MOLECULES WITHIN THE CELL

As frequently the biological effect obtained is a logarithmic function of dosage, one has assumed that inactivation of a single molecule inside the cell could account for the effect. If one compares the energy needed to kill an average cell (from 10^{-3} erg for *E. Coli*[56] to 10^{2} erg for spores of *Trychophyton mentagrophytes* or *Aspergillus*[121,122]) and the quantum yield for inactivation of most enzymes or viruses in vitro ($\emptyset = 10^{-2}$ to 10^{-5}), one can very roughly estimate that the order of magnitude of the number of cellular macromolecules altered during the killing process to be about 10^{5}.

It thus becomes very important to know what happens to a compound when irradiated *in vivo*, because it is well known that the conditions cannot be compared to those used in photochemical work. The example of chlorophyll is in this respect typical : if irradiated *in vitro* it is easily bleached; however if the whole chloroplast system is studied, bleaching becomes difficult but photosynthetic processes take place[125,194a]. A recent paper by KOSKI and SMITH describes the photosensitivity *in vivo* of chloroplasts of a white mutant of corn : in this case chlorophyll is synthetised but is lost upon continuous illumination of the seedling[149a].

Before beginning this discussion, we think it preferable to limit our interests to effects of light which include physiological changes (inhibition of mitosis, killing of cells) and genetical changes (chromosome damage and mutations).

Amongst the approaches used to try and discover which cellular components are altered or take part in these cellular effects, are

(*a*) Study of action spectra which indicate the chemical nature of chromophores involved.

(*b*) Study of biological properties of cellular proteins and enzymatic systems before and after irradiation *in vivo*.

(*c*) Direct cytochemical investigation.

(*d*) The possibility of indirect effects must also be investigated.

(*a*) *Action spectra*

To summarise the most important data, one can state that action spectra concerning *inhibition of mitosis* (micro-organisms, fertilised sea urchin eggs after irradiation of spermatozoa, starved *Paramecia*) have a very definite

nucleoprotein character (maximum near 260 mμ[19,93,94,166]). However if sea urchin eggs or well-fed *Paramecia* are irradiated, the maximum is shifted to 280 mμ (GIESE[93]), indicating the probable rôle of cytoplasmic proteins. Mutations in the fungus *Chaetomium globosum* are induced about twice more efficiently at 280 mμ than at 265 mμ (MCAULAY[175]). A small maximum at 280 mμ besides the major one at 260 mμ in the inactivation spectrum of dried T_1 bacteriophage has also been described (FLUCKE and POLLARD[81]). In the case of chick fibroblast tissue cultures, the maximum is very wide[173]. It may be important to emphasize that GIESE found with Paramecia that irradiation at 280 mμ gave less permanent effects than if 265 mμ was used[93].

In the case of killing effects, mutations, and chromosome aberrations, the action spectra are generally typical of nucleoproteins; in some instances as in tobacco mosaic virus[119a] or *Rous Sarcoma*[227a], the maximum is wide and badly defined. In the case of the tobacco mosaic virus, the action spectrum fits closely the absorption spectrum, which may mean that a quantum absorbed anywhere in the virus molecule is liable to cause inactivation.

When one interprets these action spectra, one must remember the advice of caution given by LOOFBOUROW in his 1947 review of the subject, and the technical difficulties involved in the measurements[166]. However, from the picture given for effects on mitosis and heredity, one can hardly avoid the conclusion that nucleoproteins are very probably involved. Other important cell constituents having an absorption maximum in the same region of the spectrum do exist, and it would be a useful thing to discover their localisation and concentration inside the cell. The possibility of photochemical activity of coenzymes must not be overlooked : changes in oxydo-reduction potential not accompanied by photodecomposition might be operant; previous observations by HEYROTH[114] could well be repeated and extended : this author described long ago changes in the oxido-reduction potential of uracil before any change in absorption spectrum could be observed.

On the other hand, it must not be forgotten, that light which is certainly not absorbed by nucleoproteins is also capable of inducing similar effects : Bacteriophage can be inactivated[159] and development of *Ascaris* and *Enterobia* eggs can be hindered by near ultraviolet and visible light[133]; killing of *E. Coli*[134] is also possible in these conditions; however, much higher dosages are needed and the effects are somewhat different; in the case of *E. Coli*, the lag phase characteristic of the growth of survivors is considerably increased, if it is compared to the lag phase of survivors of irradiations with short U.V. Mutations have also been observed[175].

In other cases, artificial photosensitisers may be used successfully; methylcholanthrene[120], acriflavin[189], erythrosin[138–141] may be used to

induce lethal effects in yeast and inactivation of tobacco mosaic virus or in inducing mutations in bacteria.

Acridine orange which according to the authors is bound to mitochondria can photosensitize killing of *Paramecia*[24].

Thus the cell contains, besides its essential chromophores (probably purines and pyrimidines), other chromophores capable of interfering when longer wavelengths are used. In this latter case, the photochemical reaction is oxygen dependent and responds to a mechanism essentially different to the one effective in the first case (LAURENCE[162]); more recent experiments by OSTER and MCLAREN have confirmed this[189].

It must also be remembered that a cell has to be considered in its dynamic state and as demonstrated by GIESE, the essential chromophores might be different in different physiological conditions. What happens to *Paramecia* during starvation is not clear. In the case of yeasts allowed to multiply on defiscient media, there is a loss of ribose nucleic acid[26,41,130,130a,188], and the absorption spectrum of the cell cytoplasm changes : a small maximum near 280 mμ becomes visible[41,155]. In the case of amoeba, on the contrary, ribose nucleic acid remains constant during starvation (BRACHET[29a]), probably owing to the fact that the organisms do not divide. It would be interesting to compare action spectra for different ultraviolet irradiation effects, on cells grown under different physiological conditions and where cytochemical and biochemical properties are known.

(*b*) *Effects of radiations in situ on proteins and enzymes systems*

As we shall see, micro-organisms are the most favourable biological material for this sort of study; they can be irradiated in non absorbing media and in thin layers which renders dosage estimations more accurate. Assays on proteins, enzymes or complex metabolic processes can then be readily made.

1. Numerous instances of protein resistance to U.V. have been discovered when immunologists succeeded in suppressing infectivity of bacteria or viruses without altering their immunological properties. The instance of the Tuberculosis Bacillus[215a] is well known. Furthermore, action spectra for loss of infectivity of Influenza virus type A is different to that of loss of hemagglutinising ability : in the first instance U.V. neighbouring 265 mμ is most effective; in the second, it is 280 mμ. Other viruses which have been successfully inactivated, but with no loss of immunising properties are the tobacco mosaic virus[222], Rabies[118] and *Shope Papilloma* virus[260,146].

2. *Cellular oxidations and fermentations*—It has been found that cellular respiration is not altered until a very high death rate is obtained[5,189]. But when cell division is arrested, respiratory activity is not maintained

and begins to drop after a few hours; very strong dosages are needed to inhibit respiration immediately[95]. More recently, GIESE and SWANSON have obtained a slight inhibition of exogenous respiration whilst oxidation of cellular sustrates (endogenous respiration) is slightly stimulated by moderate dosages[96].

Fermentation is inhibited in irradiated yeasts[96] and in *B. Lactis aerogenes* (DEAN and HINSHELWOOD[55]). In the latter case, similar effects have been obtained after treatment by free radicals generated by FENTON's reagent (Fe^{++} and H^2O^2), S_{32}, proflavin and the effect is probably due to mutation or perhaps to loss of adaptive enzymes[55]. Assimilation and storage of carbohydrates is also diminished : the total carbon content of irradiated cells becomes less than in the controls[96].

3. *Protein and nucleoprotein metabolism*—Nitrogen fixation in azotobacter [256a] and photosynthesis of certain algae have been found to be inhibited by moderate dosages of U.V. radiations[127].

STEPHENSON and YUDKIN[224] showed that adaptation of yeast cells to ferment galactose is still possible but decreased when growth has practically ceased after the action of U.V. radiations. More recently, SWENSON and GIESE completely inhibited the synthesis of enzymes necessary to ferment galactose after dosages leading to the survival of only 1 per cent of the cells; lower dosages lead to an increase in the time needed for galactose fermentation to begin; the increase in latent period is approximately proportional to dosage.

More recently, SWENSON has studied the action spectrum for inactivation of galactozymase synthesis[230a] and found that it was identical to a nucleic acid absorption spectrum. It is thus probable that energy is absorbed in a cellular structure containing an important proportion of nucleic acid. One could imagine a nuclear effect of U.V. light; but it would be more tempting to attribute this effect to some damage of the microsomes which are known to contain a high proportion of ribose nucleic acid (CHANTRENNE[42]). BRACHET has postulated that there is a relation between nucleic acid content and protein synthesis[26,29]; BRACHET and JEENER[26] and CHANTRENNE[42,43] have made the hypothesis that the small cytoplasmic granules might be involved in this function. This has found strong support in recent isotope work, which demonstrates that labelled amino acids are incorporated to a much greater extent in these microsomes than in any other cellular fraction[24a,50a,259a].

Another aspect of U.V. effects on nitrogen metabolism has been described by PEACOCK and HINSHELWOOD[190]. After irradiation, *B. Lactis* aerogenes loses the ability to utilise NH_3 as a source of nitrogen; however these bacteria can still continue to grow in the presence of asparagine or glutamic acid; growth is also made easier if α ketoacids (oxaloacetic, α ketoglutaric acids) are added to the culture medium. In this case, it looks as if irradiation has damaged the key enzyme systems supplying carbon

compounds which react with nitrogenous compounds to give amino acids. This damage is reversible in some of the cells which can be 'trained' by subculturing in a medium containing ammonium salts. In these conditions, the enzyme systems capable of producing α ketoacids susceptible to be aminated, recover. If recovery does not occur, this may be one of the causes of death of the bacteria.

Another example of inhibition of adaptive enzyme formation under the action of ultraviolet light has been given by ENTNER and STANIER[68], who have observed that adaptation to gluconic and to 2-ketogluconic acid of *Pseudomonas Fluorescens* grown on asparagine is prevented; under these conditions oxidation of glucose leads to a quantitative accumulation of gluconic acid which, as indicated by COHEN, leads to the inhibition of one of the mechanisms of pentose formation[51].

The inhibition of nucleic acid metabolism has not been, as in X-ray work, clearly demonstrated after U.V. irradiation; one of the most striking effects of ionising radiation is the very rapid and striking inhibition of P_{32} and labelled glycine incorporation in desoxyribose and to a lesser degree in ribose nucleic acid[1,112,124]. After U.V. irradiation of *E. Coli*, P_{32} uptake by non-viable cells (the viable cell count dropped by a factor of 10^5) is strongly inhibited (10 times less); this drop in P_{32} uptake increases with time after irradiation; two hours after irradiation, the P_{32} uptake is ten times lower still[153]. As WYCKOFF and his collaborators had found previously that 85 per cent of bacterial P is found in both nucleic acids, the metabolism of P_{32} can be considered as reflecting nucleotide changes.

These investigations had not been designed to study nucleic acid metabolism but chiefly to study bacteriophage multiplication in non growing bacteria; there is thus no specific analysis to determine what happens to desoxyribonucleic acid metabolism, which would be of primary importance on account of the striking effects of ionising radiation. However, cytochemical techniques have led to some useful informations in the hands of HAAS, WYSS and STONE. It has long been known that if a culture of *E. Coli*[90] is irradiated, part of the cells are capable of two or three divisions before degenerating; however, some of the cells become elongated (up to 150μ) and their diameter increases 2-3 fold; these filamentous forms often continue to have motility; some of them degenerate, others fragment and develop into normal looking colonies.

The last phenomenon was carefully studied[263] with the ROBINOW[200] technique, which is capable of demonstrating bacterial nuclear bodies. These elongated forms contain numerous paired nuclear bodies along the filaments. The existence of these filaments seem to indicate that irradiation first stops the ability for cytoplasmic division, although growth and nuclear divisions still seem to be possible. When these cells recover, the chromatin bodies of the elongated cells seem to undergo some sort of fusion and

rearrangements. Similar aspects have been observed by DIENES with PROTEUS[58]. HAAS and his collaborators, using different mutant species have actually shown that irradiation does lead to an increase in recombination of the genetic material of bacteria.

LEA and his collaborators[164] found similar elongated forms after cultures of *E. Coli* had been irradiated with γ-rays. The speed of elongation of these forms is similar to the speed of growth of normal cells, and LEA concluded that cytoplasmic metabolism was not inhibited; furthermore, each elongated cell is formed of long segments, responding to γ-radiation, as a single target containing unit, which might indicate that nuclear divisions have been arrested.

More recently, CALDWELL and HINSHELWOOD find no drop of desoxyribonucleic acid content of *B. Lactis aerogens* after U.V. irradiation. Elongated forms where no nuclear division has taken place, have been found to occur in these bacteria treated with proflavine[40]; and JEENER has recently obtained similar results simply by depriving cells of *Thermobacterium acidophilus* of thymidine an essential growth factor obviously needed for desoxyribonucleic acid synthesis[131].

Conversely, the British authors have evidence that only cytoplasmic fission is inhibited after colchicine treatment, and JEENER has obtained a similar effect when he grew the Thermobacteria in the absence of uracil.

4. *Viruses*—This biological material offers considerable advantages for the analysis of action of radiation : it is possible to study physical, chemical and biological properties of irradiated viruses. This has been done by COHEN with bacteriophage T_2, the autocatalytic multiplication of which can be investigated.

Normal relations between virus and bacterial host may be summarised as follows[51,52] : after infection, the bacteriophage begins to multiply after a lag of a few minutes (13 minutes in the case of T_2 phage) during which bacterial growth and certain important synthesis are discontinued (synthesis of ribose nucleic acid and adaptive enzymes). Synthesis of virus particles in the bacterial cell, is preceded, first by protein synthesis, then by the accumulation of purine and pyrimidine components. After the phage has multiplied for some time, the bacteria become lysed. The virus thus behaves like a particle capable of autocatalytic multiplication, but lacking the enzymes and energy sources necessary to accomplish this function : the enzymatic systems are to be found in the bacterial host. An infected cell can be considered as an organism the cytoplasm of which has been colonised by a foreign 'nucleus' or 'chromosome', the T phages being desoxyribonucleoproteins, probably containing no ribose nucleotides.

(*a*) *Irradiation of bacteriophage before infection* : If T_2 phages are irradiated, they become incapable of multiplying although they can still

be adsorbed on suitable bacteria[166a] and they are still capable of producing abnormal changes in the bacterial chromatin, which as in a normal inoculation becomes fragmented[167b]. If more than two particles of a suspension of U.V. inactivated phage particles (irradiated so as to leave one surviving particle in 10^4 or 10^5) are adsorbed on the same bacterial cell, they can still multiply. This *mutual reactivation* is probably due to recombination into one complete virus particle of intact genetic units from different inactivated particles. After recombination has taken place, desoxyribonucleic acid synthesis (multiplication of virus) occurs : the only difference with infection by normal particles being only the increased lag before virus multiplication takes place; during this period accumulation in the bacterial cell of purine and pyrimidine components remain normal as does also RNA synthesis, however bacterial growth and synthesis of adaptive enzymes is stopped. If several inactivated particles infect each bacteria, recombination takes place and desoxyribonucleic acid synthesis soon starts again as rapid as would be expected if the bacteria had been inoculated with normal virus. As soon as an active particle has been reconstituted, ribose nucleic acid synthesis stops as in normal infections. Let us mention that X-rays seem to induce much more permanent alteration of the virus particles, although WATSON has obtained some mutual reactivations[248a].

Everything seems to point in these experiments to the fact that normal virus when inoculated interferes with ribose nucleic acid synthesis at the expense of which desoxyribose nucleic acid synthesis can proceed; when U.V. inactivated virus is inoculated, its ability to multiply autocatalytically is lost and desoxyribose nucleic acid synthesis is blocked.

(*b*) *Infection of irradiated bacteria with normal bacteriophage*—It has been demonstrated that intact phage is able to grow on irradiated bacteria still capable of only two or three divisions[6,202] as well as on bacteria treated with nitrogen mustards[111] : bacterial enzymes remain sufficiently active after irradiation for phage multiplication : LABAW, MOSLEY and WYCKOFF have observed that even after a 10^5 drop in viable bacteria, phage can continue to multiply at essentially the same speed as in normal bacteria. Furthermore, synthesis of desoxyribose nucleic acid in the infected cell is very similar to that in the normal bacteria.

These far reaching experiments undoubtedly demonstrate that irradiation does *not* affect the enzymes of bacterial origin necessary for phage desoxyribose nucleic acid synthesis, but result in the destruction of the autocatalytic mechanism the rôle of which is to coordinate this synthesis.

This mechanism which characterises the virus particles is not hindered if the host is irradiated. However activity of the irradiated bacterial enzymes seem to diminish progressively : infection must occur as soon as possible after irradiation of the bacteria : virus multiplication becomes less

active if one waits before inoculating the irradiated *Coli*, and the more one waits before inoculation, the more one increases the lag necessary for phage growth[152a]; enzymatic activity, like in examples cited earlier decreases only after several hours, which might mean that the mechanisms responsible for enzymatic maintenance and not the enzymes themselves have been inactivated.

(*c*) *Intracellular inactivation of bacteriophage*—Another important contribution of the bacteria-bacteriophage system to facilitate the understanding of biological effects of radiations, has been the possibility of comparing the order of magnitude of the energy needed to inactivate the same substrate, in this case the virus particle, *in vivo* and *in vitro*. In addition, this work due to LATARJET and LURIA, provides an excellent method for following photochemically intracellular multiplication of bacteriophage[158,167]. Using U.V. radiations, these authors have found that only twice as much energy is needed to inactivate the same percentage of T_2 phage particles if they are irradiated immediately after their adsorption, as when solutions of phage particles are irradiated. The inactivation curve is exponential in both cases, and may be, a slight screening effect protects the intrabacterial virus. The resistance increases up to 7 minutes after infection owing, probably, to the accumulation of purines and pyrimidines in the bacteria[51]. Then the shape of the virus inactivation curve changes to a 'multi-hit curve' which indicates that phage particles are multiplying; finally the sensitivity decreases, owing probably to the transformation of the purine and pyrimidine components to active phage.

One of the conclusions which can be drawn from this work is that inactivation dosages measured in vitro do have some biological meaning and that cellular environment does not appear to change in any considerable way the appearance of photochemical effects, provided of course the molecules one is comparing are in a similar state.

(*d*) *Induction of lysogenicity*—An important aspect of Bacteria bacteriophage interaction has been discovered by LWOFF and his collaborators[168]. These authors have induced lysis in *E. Coli* K_{12} with U.V., probably by activation of a 'prophage' pre-existing in the bacteria; WEIGLE and DELBRUCK[249] have observed that the effect is obtained with dosages which usually kill 99 per cent of the phage in solution. The bacteria stop dividing and increase considerably in volume before lysis begins. LWOFF *et al.* have obtained similar effects with x-rays[168].

DIRECT CYTOCHEMICAL METHODS

Since the studies of WELS and WOTTGE describing the appearance of fluorescent substances in irradiated cells and particularly in the nucleolus of U.V. irradiated mollusc eggs and of increased light scattering when

darkfield observations were made[255,256,261], recent cytochemical methods have been used to investigate changes in cell composition during irradiation.

1. *U.V. absorption of irradiated cells*

Since BRUMBERG and LARIONOV[34] discovered that the U.V. absorption of living cells was liable to change during irradiation, several workers attempted to get more quantitative measurements.

BRADFIELD and ERRERA[30,31] described three classes of cells : those whose nuclear absorption increased (nucleated erythrocytes, fibroblasts in tissue culture), those whose nuclear absorption remained constant (mouse sarcoma, types OA and Earle HGW) and those whose absorption decreased (lymphocytes, thymocytes, bone marrow stem cells, macronucleus from *Paramecia*, epithelial cells). The effect described in the first class of cells can be ascribed in the case of erythrocytes to shrinkage of both nucleus and cytoplasm; in the case of fibroblasts, WALKER and DAVIES[247] demonstrated that a shrinkage of the nucleus also occurred and that for an incident dosage of $\sim 10^6$ erg/cm^2, there actually was a drop of 30 per cent of the total extinction of the nucleus. The disappearance of some of the nuclear absorption thus seems to be a general phenomenon; the absorption spectrum of the nucleus does not appear to be fundamentally changed, there however is a rise in the absorption minimum (at 245 mμ) relative to the maximum, but changes are too slight to be interpreted on a chemical basis. The second characteristic of the phenomenon is that it is limited to 20-30 per cent of the total absorption; when this is lost, continuing irradiation does not induce a further drop in nuclear absorption. It can also be mentioned that GERSH and CATCHPOLE[92] described a similar behaviour of fixed cells, even after fixing in formalin or absolute alcohol which probably removes low molecular weight material.

One possible explanation for this behaviour is that U.V. releases more diffusible nucleotides which pass into the cytoplasm. BRADFIELD[30] did observe a temporary rise in cytoplasmic absorption in the first minutes of irradiation, but this observation, being difficult to perform, would be worth while repeating.

If desoxyribonucleic acid was involved in this effect, one would have to admit that high molecular nucleotides are capable of passing through the nuclear membrane : chemical studies have shown that even after a drop of 70 per cent of the initial viscosity, the dialysable fraction is not more than a few per cent[70] and molecular weight of the photolysed product is still considerable[65]. This is consistent with what BUTLER and CONWAY find when desoxyribonucleic acid is degraded by ionising radiations, free OH$^\bullet$ radical or nitrogen mustards. In the case of U.V. radiation the molecular weight drops from about 10^6 to $\sim 5 \cdot 10^5$ and in the second

series of experiments a final molecular weight of $\sim 10^5$ was found[37]. Furthermore, drop of absorption due to photolysis of purine and pyrimidine rings is also improbable because, although pyrimidines are altered, the total nucleic acid absorption at 260 mμ does not change, but the absorption at 240 mμ as well as above 300 mμ does increase. A third point would be to explain why the drop of absorption only involves a relatively small percentage of total absorption, this is difficult to explain if desoxyribonucleic acid is involved, unless one assumed some sort of heterogeneity of this macromolecule. This possibility must not be rejected, on the basis of recent experiments of BENDICH[16]; this author has separated from tissues of rats injected with formate marked by C_{14}, two desoxyribonucleic acid fractions one of which incorporates 30 per cent less of the label than the other in its thymine; cytochemical evidence in support of this hypothesis has been given by BARTON[11] and enzymatic studies of CHARGAFF also confirm this[43].

The possibility that the decrease in U.V. absorption of irradiated cells concerns ribose nucleotides or other non identified absorbing component must also be considered.

That leakage of absorbing material is not only a nuclear phenomenon has been amply demonstrated by LOOFBOUROW's work which was done far earlier (summarised in [166]). Mostly, nucleotides and nucleosides of adenine, peptides and various growth factors are released in the medium by the action of U.V. If thymine nucleotides were also found, it would probably prove that during irradiation, desoxyribonucleic acid loss is actually responsible for changes in absorption.

Another unsolved problem is the reason why this effect is prevented by SH group inhibitors. BRADFIELD[30] has discussed this problem extensively, but no satisfactory reason has been advanced.

2. *Feulgen and Methyl green staining of irradiated nuclei*

Bone marrow cells have been immediately fixed after irradiation and stained with FEULGEN's or KURNICK's methyl green technique. The first stain is specific for the aldehyde group of desoxyribose unmasked by mild acid hydrolysis, and the second, according to KURNICK[151] for highly polymerised desoxyribonucleic acid, the stain needing a certain repetition of acidic (phosphate) groups to be bound. Quantitative measurements of the total dye bound to the nucleus before and after irradiation resulted in the finding that no significant change of Feulgen staining can be detected, whereas a marked drop of the affinity of the nucleus for methyl green is found (ERRERA[71]). Similar evidence has been found for cells of x-rayed grasshopper embryoes by HARRINGTON and KOZA[106]. This may be interpreted as meaning that although there is no change in concentration of desoxyribose nucleic acid during irradiation, there is evidently a change

in its macromolecular properties. We would not like to conclude too rapidly that a depolymerisation takes place; irradiation might simply result in the masking of some of the free phosphate groups by basic groups from proteins. A drop of 30 per cent in the affinity of the nucleic for methyl green occurs after a dosage of $0{\cdot}6\ 10^6$ erg/cm^2. This experiment does not completely eliminate the possibility of a leakage of purines and pyrimidines from desoxyribonucleic acid into the cytoplasm, but renders it less probable, because one would expect that desoxysugars would be involved also, which is not the case according to the Feulgen results.

One can now attempt to compare the energy needed to alter a same amount of desoxyribonucleic acid *in vitro* and *in vivo*, the drop of methyl green affinity being certainly due to its alteration. No data concerning U.V. degradation of bone marrow nucleic acid is available, so we can only compare the behaviour of marrow nuclei to thymus desoxyribonucleic acid which is far from being legitimate; however one finds that to obtain the same drop of methyl green affinity in vitro and in vivo one needs approximately 50 times more energy for the in vitro experiments. Much more work with specific nucleic acids and nucleoprotein must of course be undertaken before these experiments can logically be interpreted.

The possible biological significance of such experiments still remains to be discussed. Quantitative irradiation data on tissue culture cells with U.V., come from the work of MAYER and SCHREIBER[173]; growth of chick fibroblast tissue cultures is already slowed down by dosages between 10^4 and $2{\cdot}10^4$ erg/cm^2; up to 10^5 erg/cm^2 are needed to inhibit growth reversibly for 24 hours; for a permanent inhibition of growth one has to use $3{\cdot}10^5$ erg/cm^2. It is not certain that the conditions of irradiation used by WALKER and DAVIES are similar to those just cited, but it seems safe to say that the drop in nuclear absorption parallels the definitive inhibition of growth in the cultures. The lag described by the British authors before the drop in absorption begins, may correspond to the reversible part of the biological effect. One important fact worth remembering, is that such crude cytochemical methods as those just described, do cause visible nuclear damage for dosages comparable to those responsible for physiological and genetical cell damages.

INDIRECT BIOLOGICAL EFFECTS

(*a*) *Biological effects of irradiated media*

Many reports, mainly from CLARK, STONE, WYSS and their collaborators have described killing and mutation effects obtained by treating unirradiated microorganisms with ultraviolet irradiated broth[47,102–104,226,263]. A more recent paper by the same group states that the most effective regions of the spectrum is that below 200 mμ[104]. However, when the

253·7 mμ line of the Hg lamp is used to irradiate the medium much higher exposures are needed to get mutations by irradiating the broth alone[104,226,227]: this suggests that the rôle played by these indirect effects is not as important as the direct effects. It does however indicate that a direct action of the U.V. on the cell component is not necessary.

Some important facts discovered during these experiments may be summarised:

1. H_2O_2 action on the broth is capable of duplicating the action of radiations (U.V. or ionising) or nitrogen mustards which have also been used to treat the culture medium.

The medium treated with H_2O_2 becomes mutagen, but H_2O_2 is not the active agent, because after the medium has been incubated in its presence for some time, it can no more be detected; oxygen can still be evolved under the action of peroxydases[276]. That organic peroxydes have mutagenic properties has been demonstrated by DICKEY, CLELAND and LOTZ[57]. H_2O_2 alone does not seem to be directly mutagen in the case of bacteria[47] but it does increase the mutation rate of *Neurospora*[2] and it has a lethal action on bacteriophage S_{13} [2].

Blocking cellular catalase by KCN or azide induces a higher percentage of mutations in the absence of any other treatment; this effect may be due to the accumulation of H_2O_2 or peroxides in the absence of activity of the enzymes capable of destroying these agents[246].

2. The effect is maximum for low bacterial concentrations: if too many bacteria are inoculated there is no increase in mutation rate[132a].

3. WYSS and his group have observed that preincubation of the medium with 2 methyl 1·4 naphtoquinone and N mustards, which are SH inhibitors also increase the mutation rate. This effect not being obtained with heavy metals, the phenomenon is probably more complicated than the mere inactivation of SH groups[47a].

4. The temperature at which the broth is irradiated has an effect on death and mutation rate[103].

Although these studies have emphasised the possibility of an indirect action of U.V., it is a pity that attempts to find out the nature of the mutagens produced in the culture medium have not been made since the preliminary experiments[226] in which different components of the medium were irradiated separately; and it would also be instructive to find out the action of irradiated bacterial homogenates. Whatever be the active substrate induced in the medium, the mechanism of its action remains the major problem and the possible occurrence of active substrates inside the cells awaits further experiments.

The possibility of an indirect effect seems however to be confirmed by a recent investigation of THOMPSON *et al.*[237] who found that bacteria are protected by pyruvate against the lethal and mutagenic action of ultra-

violet, x-rays and also H_2O_2; the pyruvate must be present during the irradiation. However in the case of ultraviolet irradiation, part of the protective effect is due to screening and quantitative data are needed to appreciate the part of this protection, possibly through the reaction of pyruvate with peroxides or H_2O_2.

(*b*) *Other possibilities of indirect mechanisms*

1. The possibility of hydrogen peroxyde, not necessarily produced by the action of irradiation, taking part in photobiological processes must not be overlooked, for other reasons than those which have just been developed. It has been shown by BUTLER and by HOLMES and their collaborators that compounds like desoxyribonucleic acid[36] or ribonuclease[123] are very sensitive to the action of ultraviolet light when irradiated in the presence of moderate amounts of H_2O_2 (10^{-3}M). These concentrations of H_2O_2 increase considerably the speed of degradation of the macromolecules, owing undoubtedly to the action of OH• radical known to be liberated in these conditions by the action of U.V. light on H_2O_2.

Although oxygen peroxyde has been stated not to accumulate in cells owing to the ever present activity of catalase and peroxidases (BALDWIN[83]), it has been found to be formed in appreciable amounts in certain cases. It might be worth while investigating if this accumulation has any relation to increase in U.V. sensitivity of these organisms. It would be possible to irradiate cells whilst these are cultured in conditions where peroxyde is known to accumulate. Oxydation of most amino acids is catalysed by a flavine enzyme with the production of H_2O_2; if catalase were blocked by suitable inhibition, H_2O_2 might accumulate. Accumulation of H_2O_2 (0·1 μ mole/ml) is known to occur in washed sperm suspension[242,244] and also when anaerobically grown cells of *Steptococcus Faecalis*[208] are supplied with oxygen* and probably during butyrate oxidation in the absence of inorganic Phosphorous by KLOSTRIDIUM KLUYVERI[145]. The concentrations of H_2O_2 formed in these cases would probably be sufficient to considerably increase the action of ultraviolet rays. The activation of non-irradiated bacteria under the action of catalase as observed by MONOD, TORRIANI and JOLIT[184b] does seem to indicate a possible accumulation of H_2O_2 when the cells are grown on a deficient medium.

2. Another possibility difficult to estimate at present, might also be the occurrence during irradiation of reactions similar to the ones listed on page 92. It is of course not possible to discuss these cases, but to take one example, quite appreciable amounts of iron exist in living cells: guinea pig mitochondria are stated to contain 4 per cent ash and approximately 0·03 per cent iron[59]. Although this is probably bound in organic molecules, reactions of the type mentioned must be disproved experimentally before being

* These cells have been shown to contain more riboflavin when grown aerobically (209).

forgotten. Pigments like hematines, porphyrines or flavines known to be good photosensitisers (BLUM[18]) do not have absorption spectra resembling action spectra of the biological effects we made reference to earlier, but they may be responsible for photodynamic effects obtained in other spectral regions than those corresponding to proteins or nucleic acids.

Effects of oxygen and temperature—Although oxygen is not necessary for U.V. induction of killing and mutation effects[95] recent data of HEINMETZ and TAYLOR do indicate that in the frozen state (—70°C) *E. Coli* are more sensitive to U.V. irradiation in the presence of O_2 than in N_2; the cells are still more sensitive when irradiated in air at 25°C, but differences in light scattering make accurate actinometric determinations difficult. In the frozen state, change in *p*H has a definite effect, the cells being killed more rapidly in the alcaline region. These studies do indicate effects of oxygen tension and temperature, and photobiological investigations similar to those of HOLLAENDER on the effects of ionising radiations on bacteria cultures and irradiated in different conditions of O_2 tension would certainly help to clarify the problem of respective importance of direct and indirect effects[122a].

PHOTOREACTIVATION

One of the most promising fields in the study of biological effects of ultraviolet light, is the now well established possibility of reversing these effects by strong visible illumination. Reactivation by light has been studied by PRAT and others[192,199,257] but interest in the field has developed considerably since KELNER's classical paper. Photo-reactivation has now been obtained for the following effects.

1. *Inhibition of mitosis* : in *Paramecia*[147] and increase in the time needed for the appearance of the first cleavage furrow in *Arbacia* eggs[20,21,172]. In the case of *Paramecia*, no significant difference in reactivation were noted if U.V. irradiation had occurred at 265 mμ or 280 mμ.

2. *Killing of micro-organisms: E. Coli, Neurospora, Penicillium, Saccharomyces*[142,186,97,33] and inactivation of Bacteriophage[63]. However, reactivation of Riboflavin photosensitised inactivated T_{6r} phage has not been possible (GALSTON[87]).

3. *Mutations in micro-organisms*: *Neurospora*[98], *E. Coli*[142,185]. If photoreactivation of killing and mutation effects are parallel in the same organism, one would have a convincing argument to support the view that both effects are due to a single initial process. Although GOODGAL[98] has found such a parallelism to exist for both effects in the case of *Neurospora*, this parallelism is only partial in the case *E. Coli* (BROWN[33]); reversal of both effects occur for low dosages, but mutations become

irreversible if a certain dosage of U.V. is exceeded (NEWCOMBE and WHITEHEAD); this leads different authors to postulate the induction of a photostable and a photosensitive mutagene[63,147,186].

Only zero point mutations are liable to be photo-reversed; end point mutations are not reversed[33].

4. *Morphological effects on cell nuclei*: inhibition of the appearance in the macronucleus of granular structures which finally unite and vacuolise[147], changes induced in nucleoli appear also to be photo-reversed (CARLSON and MCMASTER cited by KIMBALL and GAITHER[147]).

5. *Enzymatic adaptations*: recovery of galactozymase synthesis in yeast (SWENSON and GIESE[231]). Inhibition of galactozymase activity by U.V. is not possible once the adaptation of the cells is complete and irradiation is most effective if applied early. Dosages needed for inhibition of adaptative enzyme formation are relatively high: many cells are killed and inhibition of adaptation occurs in the survivors. Both effects seem to result from different cellular damage: photo-reactivation of adaptive enzyme formation is more efficient than reversal of killing effects.

6. *Induction of bacteriophage formation in lysogenic bacteria* (LWOFF *et al.*[168]). This phenomenon is photo-reversible[129] even if the induction has been caused by ionising radiation[157].

We will now try to summarise the behaviour of these processes of photo-reactivation; possibly future experiments will tell us that these facts must not be generalised.

When nuclear components are the chief ones affected as in T_2 bacteriophage inactivation[63] and in cleavage delay induced by irradiating *Arbacia* spermatozoa[20,21,172] reactivation only takes place when cytoplasmic material is present, that is when infected bacteria or when the zygote are illuminated (reactivation becomes possible 7-9 minutes after fertilisation[172]); as suggested by BLUM, it would be tempting to associate the possibility of reactivation with some event connected with nucleoprotein reduplication. So far (DULBECCO) no reactivation of phage has been obtained in the presence of homogenised bacteria; integrity of the cytoplasm is necessary and inhibition of adsorption of the phage particles also prevents reactivation.

Illumination must be applied promptly after irradiation; if it takes place before irradiation[172,20], it is useless, and reversibility decreases rapidly when time between U.V. and visible light is increased.

Lowering the temperature or slowing down the metabolism by using a poor culture medium (*E. Coli*) increases the time one can wait before illuminating; bacteria irradiated at —55°C and kept for six days at —10°C can readily be reactivated[109]. In the case of bacteriophage T_1: the possibility of reversing U.V. effects begins a few seconds after adsorption and remains constant for the first 15 minutes of intrabacterial development;

after 20 to 30 minutes, it becomes impossible, unless bacterial metabolism has been slowed down by depriving the culture of essential metabolites; when this is done, one can wait for several hours before the capacity for reactivation is lost[142].

The per cent reactivated organisms is much higher for low dosages of U.V.; this is true for bacteriophage[63], *E. Coli*, and *Paramecia* where high dosages are able to kill irreversibly the organisms before the first division takes place[147]. When low dosages are used, bacteria irradiated a second time after a first reactivation continue to retain the ability of being reactivated[185].

The proportion of cells reactivated by visible light is apparently independent of U.V. dosage when mutation and killing effects are studied on *E. Coli*[149].

Mechanisms of photo-reactivation

1. Photo-reactivation is apparently a process having a *high temperature* coefficient[63]: it does not take place when illumination occurs at below freezing temperatures (—50°C); furthermore at such low temperature, long U.V. light (300-400 mμ) kills the bacteria[109].

Oxygen does not appear to be necessary in the cases studied: inactivation of bacteriophage[63], killing of *E. Coli*[132]; photo-reactivation is not prevented by KCN[63].

2. The action spectra obtained for photo-reactivation indicates that in different cases, different receptors are active. Reversal of inactivation of T_1 phage[63] and killing of *E. Coli* (KELNER, cited in [185]) is more efficient if light of 370 mμ is used; in the case of *Streptomyces griseus*, 436 mμ is most effective: it may be significant that this last region is that of the Soret band of porphyrins. In the case of bacteriophage T_1, there appears a slight rise of absorption between 300 and 360 mμ after irradiation. This small maximum could be displaced to 370 mμ when the phage is adsorbed to bacterial components; a rise in absorption of several irradiated proteins[176] and of desoxyribonucleic acid[166,170] in that region of the spectrum is not uncommon after U.V. irradiation.

In the case of *Arbacia eggs*, BLUM and MARSHAK have given evidence that the chromophore is not related to the cytoplasmic pigment of the embryo[20] and MARSHAK has found that the wavelength 330—470 mμ are more effective than longer ones and that the presence of riboflavin has no effect[172].

3. Reactivation is logarithmically proportional to the duration of illumination in the case of T_1 phage[63]; this probably indicates that one photon is capable of transforming an inactive particle to an active one.

4. NOVICK and SZILARD[186] have developed a theory in which light reactivation for the killing and mutagen effects of U.V. on *E. Coli* is

assigned to the action of a photosensitive poison, the amount of which present in the bacteria, when they are allowed to multiply, determines the number of survivors or mutants. NEWCOMBE and WHITEHEAD imagine the existence of two poisons, one of which is not photosensitive and is responsible for the part of the effect of U.V. which cannot be reversed by visible light. However, a few of the facts described previously and which are common to a variety of materials, make it very plausible that the mechanism of photo-reactivation is quite a general one although the photo-receptors may be different. If this be the case, it is difficult to avoid the conclusion that when bacteriophage is inactivated, the 'poison' induced by U.V. light is the inactivated phage particle itself: one could easily imagine that part of the effect, perhaps ruptures of peptide bonds is irreversible, whilst other rearrangements might be less definitive. So far DULBECCO's hypothesis of the formation by U.V. of 'inhibitor molecules' in the phage particle, thus blocking the possibility of phage growth remains the one best capable of being suitable for generalisation: this inhibitor could be dissociated during illumination; a dark reaction involving some bacterial receptor could finally be responsible for the removal of the inhibition. It might be of interest to find out whether photo-reactivation is a phenomenon related to reduplication of nucleoprotein; the U.V. induced cell lesions so far reactivated seem to be related to such processes and it might be interesting to see if effects like changes in cell permeability or cytoplasmic streaming are also liable to be reversed by visible light.

5. A final aspect of photo-reactivation lies in the low yields observed when x-rays instead of U.V. are used to induce the effect. Most effects of U.V. cited earlier in this chapter have also been produced by x-rays and attempts for photo-reactivation have been fruitless or very poor (KIMBALL, BLUM, NEWCOMBE, DULBECCO). However, LATARJET[157] has recently observed that lysogenicity induced in *B. Megatherium* by x- or U.V. radiation[129] can be prevented by the action of visible light. It would be of interest, owing to the importance given more and more to x-ray indirect effects, to investigate the possibility of reversing by illumination the effects of different cell poisons.

As GIESE emphasises[94], it may be difficult to admit a photo-reversal of peptide bond splitting; perhaps milder changes in the inactivated substrates, like those involving H bonds or new cross linkages could perhaps be considered more favourably. The rôle played by cytoplasmic factors will also have to be investigated.

Photo-reactivation of enzymatic systems

From what has just been discussed, the mechanism of the photo-reversal of biological effects of U.V. light is not understood. It may however be

of interest to briefly summarise what is known about activation of enzymatic systems by light. We shall only mention for reference, the well known case of the reversal of respiration of cells, intoxicated with carbon monoxide* (WARBURG and NEGELEIN[247a]); this led to the discovery of respiratory pigment and has now become classical textbook biochemistry[8a]. We have already mentioned the case of reactivation of spontaneously oxidised triosephosphate dehydrogenase by near ultraviolet in the presence of reduced diphosphopyridine nucleotide, its normal coenzyme[212], and the photo reduction of di- and triphosphopyridine nucleotides in the presence of illuminated chloroplast preparations : this reaction regenerates reduced coenzymes which can thus be coupled to other reactions (reductive fixation of CO_2 by pyruvate and reduction of pyruvate to lactate[240,241,245]).

We might still mention the photo-stimulation of methylene blue reduction by yeast enzymes[105]. More recent experiments by BERNHEIM and DIXON describe a similar effect of illuminated preparations of milk xanthine oxydase. Short exposures to light give a stimulation of enzymatic activity in the presence of eosin or methylene blue (even if KCN is present); but longer exposures result in inactivation of the enzyme. The effect appears to be due to the photochemical production of hydrogen peroxyde, which even in very low concentration (10^{-6} or 10^{-8} m) may have an effect in the absence of light[17].

Another example has been recently described by GALSTON, who found that indole acetic acid (IAA) oxidase may be photochemically activated by visible light, the action spectrum being that of a flavin[87]. This is not surprising in view of the fact that IAA oxydase is known to be a flavoprotein. However, when studied in the dark, these preparations may also be activated by dialysis, which means that both light and dialysis result in the removal of an inhibitor of enzymatic activity : GALSTON concludes that light activation is probably due to the reversal of a naturally occurring inhibition. According to the idea developed by GALSTON, the oxydation of IAA probably involves two steps : the first one, a light activated production of H_2O_2 by the flavoprotein and the second, an oxydation of IAA by the action of peroxydase. The light activation of the flavoprotein or its dialysis results in the production of more H_2O_2 (in the last case by removal of an oxidisable substrate) which thus is no more in limiting concentration for the oxydation of IAA by the peroxydase.

Another reactivation process, not mediated by illumination, might finally be worth mentioning : it concerns reactivation of chloroplast preparation, by reaggregation of disrupted particle by the action of methanol[184]. As many instances of irradiation induced enzymatic damage do probably concern systems bound to cytoplasmic particles, these experi-

* Similar experiments have been recently done by BÜCHER and KASPERS on carboxymyoglobin; they found that carbon monoxide was oxidatively split from the hemin pigment and that light absorbed both by the hemin and by the protein chromophores was efficient. (34a).

ments suggest a way of approach to the study of reactivation of complex enzyme systems.

Influence of temperature after irradiation

The possibility of diminishing effects of U.V. or ionising radiations by storing the cells at low temperature (+5°C) is not new and is efficient in the case of *Ascaris* eggs (x-rays, COOK[53]) and micro-organisms (U.V. and x-rays; LATARJET[156]). These effects of low temperature may be interpreted as the inhibition of 'dark' secondary reactions. CLARK has summarised earlier data on the subject[45,46].

Quite a different mechanism appears to be operant in the case of *heat* reactivation of U.V. irradiated micro-organisms. If *E. Coli* are irradiated and then incubated at different temperatures, the number of surviving cells increases if the temperature is raised from 38°C to 43°C (STEIN and MEUTZNER[223]). ANDERSEN who has also studied this effect[3,4] has observed an identical behaviour with *E. Coli B*, but not with the B/r strain : of 10 strains of *E. Coli* tested, only two did respond to heat reactivation. This suggests that the mechanisms for heat and for light reactivation are different, because all strains so far studied may be photo-reactivated; another difference between the two mechanisms is that x-rayed *E. Coli B* can be partly heat reactivated. Another case of heat reactivation is that of T_1 phages, which as in photo-reactivation do not respond unless they are adsorbed on the appropriate host (*E. Coli B*) (BRESCH[32]). In all these instances, reactivation is of course never complete; but it is interesting to note that as in T_1 reactivation, it may take place at temperatures high enough to have noxious effects on unirradiated phages. It may be noted that aged triosephosphate dehydrogenase can be reactivated by heating[195a].

Reactivation by catalase

MONOD and his collaborators have been able to prevent up to 20 per cent of the lethal effect of ultraviolet rays on bacteria irradiated in citrate buffer by culturing them after irradiation on a medium containing catalase or peroxydase; ferrous sulphate, known to decompose H_2O_2 into free radical which could be 'neutralised' by the culture medium is also active.

CONCLUSIONS

When one considers biological damage caused by radiations from a cellular standpoint, one is confronted with two possibilities. Either the chromophores themselves belong to structures carrying out specific functions and existing in the cell as limiting factors; or these specific factors are altered under the influence of toxic chemicals, produced by the action of radiations on less important cellular constituents. These may modify physical and chemical properties of cellular structures or compete with normal

cellular substrates and build up abnormal constituents. The problem of how radiations act on cells thus becomes the one of deciding what is the relative importance of these possible mechanisms.

Considered from a dynamic point of view, the cell contains in its nucleus, hereditary factors, the genes, capable of multiplying autocatalytically. These exert their control over cytoplasmic activity and probably over synthetic processes, which involve the participation of particles : mitochondria, microsomes[25,50] and perhaps smaller ribonucleoproteins still[232]. These particles are probably also capable of multiplying autocatalytically[27,48,130a] and it is not impossible that one of the functions of the genes is to control this multiplication : a certain number of specialised particles (Kappa particle of *Paramecia*[216]) plasts in green plants[197] only multiply in the presence of certain genes. Furthermore, the loss of the X chromosome of Drosophila larvae results in the inactivation of cellular respiration[22] probably owing to the impairment of the cytochrome-cytochrome oxydase system, which is known to be located in the large cytoplasmic granules[119]. The relationship between genes and the formation of specific enzymes capable of simple reactions is beginning to be understood (BEADLE, TATUM[13]). In addition, certain facts support the idea that the cell nucleus performs some less specific functions : it is in close contact to chromatin that the nucleoli appear, and cytochemical investigations[29] have shown these to contain ribonucleotides, a small proportion of which are in a low molecular state[244a]. It becomes probable, as BRACHET has suggested[29], that these oligonucleotides are the origin of cytoplasmic coenzymes, necessary for storage of energy (phosphorylations); this would mean that the nucleus has a remote control of all endergonic reactions.

Cytoplasmic particles are only beginning to be known[48]; many arguments have accumulated to support the idea that some of them (microsomes) are involved in protein or enzyme synthesis[29,43], that others (mitochondria) are the support of cellular oxidations (Krebs cycle, oxidation of fats) coupled to the synthesis of high energy phosphate bonds[100].

One is beginning to have some indications concerning the structure of these cellular particles : mitochondria are cell organelles containing in addition to protein, phospholipids and small amounts of ribose nucleic acids, pigments (cytochrome C) riboflavin and the dehydrogenating enzymes we have just mentioned, the activity of which is dependent on the presence of di and triphosphopyridine nucleotides. The microsomes contain a much higher percentage of ribose nucleic acid and probably some cytochrome reductase, also active in the presence of diphosphopyridine nucleotide[119]. We have already mentioned that the inactivation of adaptive enzyme formation has an action spectrum suggesting that the chromophore is a nucleoprotein : the inactivation of these granules might be the cause of this inactivation, although nuclear damage (a mutation)

cannot be dismissed. As we see, these particles contain many suitable pigments which are capable of acting as photosensitisers. It is generally accepted that these cytoplasmic 'genes' are much more numerous than nuclear genes (SPIEGELMAN[218]); this suggests that the functions they perform could be less sensitive to radiation damage. There are however a certain number of arguments supporting the idea, that at least in special circumstances, the opposite hypothesis is true. If *Euglena* are grown in conditions such as their multiplication is accelerated, one soon reaches the moment when only of few or even no chloroplasts can be seen in the cytoplasm; these particles having a rate of division slower than the whole *Euglena*. Grown again in normal conditions, the descendants will be seen to have a normal number of plasts (LWOFF[50]). However, if these few chloroplasts are inactivated (by irradiation or any other suitable means), this will result, if the organism is an absolute autotroph, in a lethal mutation.

One expects similarly, that it would be easier to inhibit galactozymase synthesis in yeasts, if these have been allowed to multiply actively in the absence of galactose, in which case the cytoplasmic particles responsible for this synthesis appear to be diluted out or even lost[219].

The importance of some of these specific cell components in radiobiology comes from the following experimental facts.

1. The wide variety of sensitivity displayed to radiations by different micro-organisms had received no satisfactory explanation until WITKIN[258] discovered the remarkable fact that a certain strain of *E. Coli* (Strain B) is liable to undergo spontaneous mutations to the B/r strain much more resistant both to ionising and U.V. radiations, as well as to various chemical agents (N mustards, H_2O_2, penicillin, sulfamides). Killing is exponential for both strains when X-rays are used; in the case of U.V., the killing of *E. Coli B* follows an exponential relation until 99 per cent of the bacteria are inactivated; if irradiation is continued, a sharp inflection of the curve indicates that the survivors are more resistant[56,66].

When the B/r strain is irradiated, a sigmoid curve is obtained, indicating a 'multi hit' effect. LATARJET and DEMEREC have calculated that the U.V. inactivation corresponds to the necessity of 3 hits. Recently, CARTER and MORSE[184c] have found 3 to 4 times more desoxyribonucleic acid per cell in the B/r strain, which supports the view that this strain is polyploid. More recently, LATARJET and EPHRUSSI[160] have found an increased resistance to irradiation of a diploid strain of *S. Cerevisiae* which responded to ionising radiations by a '2 hit' inactivation curve; the corresponding haploid strain responded logarithmically. In the case of U.V. radiations, CALDAS and CONSTANTIN[39] have described a similar phenomenon. In the case of haploid cells, the effects observed 20 hours after irradiation appear to be irreversible (immediate or delayed death); in the case of diploid

yeast, frequent restaurations may be seen : chains of giant cells are capable of giving rise to normal colonies; this phenomenon seems very similar to the recombination figures observed by WYSS and his collaborators[263] when long forms of *E. Coli* are observed cytochemically. Similar effects of polyploidy in the case of U.V. and ionising radiations have been observed by others[66,238] and have also been found for insects (HABROBRACON[44]). Studies of induction of mutations and chromosome breakage are too well known to discuss them here, but both cytological and action spectra data do indicate of definite effect of U.V. on nucleoproteins[221,228].

2. That U.V. or X-rays are capable of inactivating intracytoplasmic cell particles has been demonstrated by LURIA and LATARJET who irradiated phage in infected bacteria[157,167]. Other indications come from the work of PREER who inactivated (with X-rays) the Kappa particles of *Paramecia*[193,194]. The inhibition of photosynthesis obtained by U.V. irradiation of algae[127] is evidently due to chloroplast damage, and it is significant that, when radiations inactivate enzymatic systems, they are generally complex structures like those responsible for the synthesis of adaptive enzymes or nucleic acid. If however enzymes are found to be radioresistant (respiration and fermentation), the mechanisms responsible for the maintenance of these activities seem to be impaired.

It may be interesting to note that some effects of moderate irradiation like delay in cleavage of eggs[9,49] or inhibition of adaptive enzyme formation[218] in bacteria have been observed after intoxication of the organisms by drugs like dinitrophenol which are known not to inhibit cellular respiration but which prevent phosphorylations and so diminish the cellular reserves of chemical energy. These systems are bound to mitochondria and their alteration might explain some of the effects of ultraviolet radiations; in starved organisms[94] the maximum of the action spectrum has shifted to 280 mμ indicating a high relative participation of cellular proteins. The mutagenic effects of low dosages of U.V. has also been increased by dinitrophenol intoxication of spores of *Aspergillus Terreus*[229], even if the toxic agent is allowed to act after irradiation; no such effect of dinitrophenol is observed in the case of the killing effect.

It may be of interest to remember that the number of mutations in micro-organisms increases considerably if the cells are allowed to undergo several generation cycles before being assayed (end point mutations[56]). The effect may be due to 'diluting out' of the remaining active genes, which might be lost in some of the descendants.

An interesting approach to the investigation of the respective rôle of cytoplasmic and nuclear factors in irradiation effects has been attempted by MAZIA and HIRSHFIELD[174]. The major effect of low dosages of U.V. on nucleated half amoebae or whole amoebae is the delay in division;

stronger irradiations inhibit completely all divisions but the cells survives for quite a long time. When half of the cytoplasm has been removed, the amoebae are much more sensitive to all these effects, and the survival time is very much reduced. If non-nucleated halves are irradiated, their cytoplasm disintegrates after a certain time, even for dosages that have no effects on normal amoebae or only slightly delay the devision in the nucleated halves. These experiments suggest that both the cytoplasm and the nucleus are sensitive and that both have a protecting effect on the other part of the cell, perhaps by enhancing recovery mechanisms. The study of these amoebae by cytochemical methods similar to those described by BRACHET[29,29a] would be the next step in the study of these phenomena. It is known that phosphorylations are practically completely arrested in the non nucleated fragment[173a], which makes it obvious that no recovery can be expected.

The specificity of desoxyribonucleic acid

We would like to conclude by some considerations on the specificity of desoxyribonucleic acid and its probable rôle in irradiation damage of cells. Since AVERY'S[8] penetrating study on the possibility of inducing mutations in Pneumococci by the action of highly polymerised desoxyribonucleic acid, many reports have confirmed this finding[23,233], and the existence of several transforming principles, active at very low concentrations has been established (TAYLOR[233], HOTCHKISS[128]).

THOMAS[236] has been led recently to postulate that the specificity of desoxyribonucleic acid might be due to the existence of very labile bonds, like hydrogen bonds, which are very sensitive towards mild chemical agents (moderate changes in H^+ concentration). We were led to postulate that rearrangement of such bonds might be one of the causes for the apparent radio-resistance of desoxyribonucleic acid. The *in vivo* irradiations of cells by ultraviolet or ionising radiations induce an immediate change in the macromolecular[69] or straining properties of this nucleic acid[71,106]. That these crude methods of investigations are sufficient to demonstrate such changes with dosages within the limits of those used to obtain killing effects on similar cells suggests that less drastic, but perhaps just as specific changes of this nucleic acid, are obtained for smaller dosages still.

The alterations postulated here may of course be due to direct photolysis of the nucleic acid, but other indirect alterations must be considered: cross linkages due to the action of free radicals, or induced cell poisons (perhaps peroxydes), of the types of those discussed by GOLDACRE *et al.* and by ROSS, are far from being improbable. Hydroxyl radicals could induce the formation of other radicals which would become reactive, or as it might be the case for mitotic poisons, the poison molecule itself could be

the linking agent[96a,201]. In this respect, increase in effectiveness of U.V. radiation by N mustards is of interest[230].

That damage to the desoxyribonucleic acid complex is a probable cause of important cellular effects rests on the following experimental facts.

(*a*) Bacteriophage of the T series, which may be considered as a pure desoxyribonucleoprotein, have to be directly irradiated to be inactivated; active phage particles infecting irradiated bacteria may still multiply[51,52,152,153].

(*b*) HERSHEY, KAMEN[110] and their collaborators have seen that Bacteriophage T_4 containing P_{32} in its nucleotides becomes inactivated faster than would be expected if this was only due to the β radiation emitted by the radioactive isotope : transmutation of P_{32} to S_{32} leading to virus inactivation is the other possible alternative, and this can only be due to loss of specificity of the nucleotide moiety of the virus.

In the case of bacteriophage, it is thus the destruction of the system capable autocatalytic multiplication, which is responsible for the lethal mutation.

If direct alteration of desoxyribonucleoprotein or other cellular particles do take place, this does not necessarily mean that it is the only possible cell damage : a living organism is such a complex system that it is very likely that physiological changes are the result of the alteration of a whole series of mechanisms the interdependence of which is not yet understood. To consider once more the case of desoxyribonucleic acid, this final instance is again instructive : Thermobacterium acidophilus is a microorganism for which thymidine, a specific pyrimidine riboside of desoxyribonucleic acid, is an essential growth factor. Deprived of this building block, the reduplication of the bacterial nuclear bodies becomes impossible: and the bacteria cannot continue to multiply although they grow into long filaments, like has often been observed after radiation or other damage. Similarly in the absence of uracil, ribonucleoproteins fail to be synthetised (JEENER[131]). An interference with the integration of these normal constituents in important cell structures is also a cause for physiological disfunction. If this, as we have seen earlier, may result from an alteration of the macromolecule which organises this synthesis, it can just as well be due to a competitive action of slightly changed metabolites leading to the edification of a new but unphysiological structure.

The case of desoxyribonucleic acid has been discussed here because it probably is better understood than others which may be just as important. The understanding of physiological effects of radiations has now become a problem of finding out, which are the different cellular structures involved, the mechanisms of these alterations and their relative importance.

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ADDENDUM

Recent experiments of LATARJET and MILETIĈ (personal communications of results to be published in *Annales de l'Institut Pasteur*) have shown that both near U.V. and visible light are capable of forming in broth and, to a smaller degree, in agar, certain substances sensitive to catalase. These are injurious to T_2 phage suspensions and have no effect on bacteria alone (*E. Coli* B and K 12). Effects of U.V. on *E. Coli* B may thus be reversed even if illumination takes place in broth. However, the illuminated phage-bacteria complex is sensitive to long U.V. and visible light, chiefly during the first stages of intracellular development of the T_2 phage. Therefore, when illuminated in broth there is no photo-reactivation, but a lethal effect on the bacterial virus ; photo-reactivation does occur if the infected bacteria are illuminated in a mineral medium. Finally, the lysogenic strain, *E. Coli* K_{12}, reacts to visible light in the following way, when growth of the bacteria is tested :—

(*a*) when lysogenic induction is possible, there is a photo-reactivation by illumination in a mineral medium, but a lethal effect when in broth.

(*b*) photo-reactivation in both media, when no lysogenic induction is possible.

6

RECENT WORK ON THE APPLICATION OF THE THEORY OF THE IONIC DOUBLE LAYER TO COLLOIDAL SYSTEMS

F. Booth

1. *Introduction*

THE CONCEPT OF AN electrical double layer at surfaces separating liquid and solid phases seems to have been originally put forward by QUINCKE[1] as long ago as 1861. At that time it was pictured as two parallel layers of electricity, each of the same uniform charge density but of opposite sign, separated by a small distance. The classical paper of HELMHOLTZ[2] on the electrokinetic effects used this model. Today it is believed that the real state of affairs at phase interfaces is far more complex. For example, if we consider the boundary between a solid and an aqueous solution containing electrolyte, the potential changes as we move from the interior of the liquid normal to the interface, may well be as shown in Figure 1. The total potential difference between the solid and liquid phases is made up of two parts, one lying in the solid phase, the other in the liquid. In the figure both contributions have been drawn with the same sign, but in practice it may happen that the two contributions are of opposite sign.

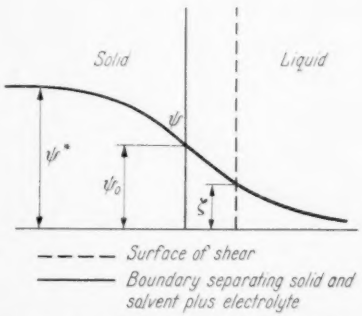

Figure 1. Potential variations at the solid-liquid interface.

It is natural to ask how the curve of Figure 1 is obtained since there is no direct method of following the potential changes in the boundary region, which may only be a few Ångstrom units thick. There is even no general method of finding the *total* potential difference ψ^* between the two phases. The main evidence for the form of Figure 1 is, however, very briefly, as follows. It is possible to form some estimate of the contribution ψ_0 of the liquid to the total potential from the experimental data on the so-called electrokinetic effects. These involve the streaming of the liquid past the solid boundary. From the data it is possible to deduce a well-known quantity called the zeta-potential, ζ. This may be defined as the

potential difference between the interior of the liquid and the surface of shear at the boundary, or in other words, the surface where the material becomes effectively rigid. This definition presupposes that all relevant physical quantities like the dielectric constant, density and viscosity of the electrolyte retain their macroscopie values right up to the surface of shear. It is also possible in some cases to estimate from thermodynamical theory how the total potential ψ^* changes as the composition of the solution is altered. In general it is found that ψ^* and ζ behave quite differently. Hence if it is provisionally assumed that ψ_0 and ζ may be identified, it follows that ψ^* and ψ_0 cannot be set equal to one another. Of course it may not be legitimate to identify ψ_0 and ζ. For it might happen that the boundary between the two phases in the chemical sense is not quite the boundary in the physical sense. For, owing to the strong intermolecular forces at the boundary there may be a thin layer of matter which is effectively rigid although chemically it belongs to the liquid phase. Another possibility is that the properties of the liquid undergo continuous variations as the interface is approached. In this case the potentials ψ_0 and ζ, as it is usually calculated, will be related in a very complicated fashion. Thus we see that, unlike ψ_0 and ψ^*, the zeta-potential may or may not have a precise physical significance; for this reason it is better not to identify strictly the two potentials ψ_0 and ζ.

In the solid region, where there is a contribution to the potential change, a region sometimes known as the *compact* double layer, the potential variation may arise from a variety of causes; for example, adsorbed ions or dipolar molecules, or in the case of salts, interstitial ions. In the liquid region however, the potential variation is always due to the same mechanism; it arises from an excess of ions of one sign and a deficiency of ions of the opposite sign, both excess and defect being with reference to the concentrations in the main bulk of the liquid. This part of the boundary region is known as the *diffuse ionic* double layer and it is with this that we shall be mainly concerned in this article. The theory of the compact double layer is much less developed than that of the diffuse double layer. The best known theory, that of STERN[3] is based on the hypothesis of ionic adsorption. More recently MOTT and GRIMLEY[4] and GRIMLEY[5] have presented a different interpretation applicable to ionic crystals in contact with electrolytes. Other writers, for example GRAHAME[6], have given more complicated models of the compact double layer in which the finite size of the adsorbed ions is taken into account.

So far we have only considered one type of interface, but similar effects occur with other phases and materials. For example, near the boundary layer between an oil and an aqueous solution, there may be potential jumps due to diffuse ionic double layers in both the liquid phases together with a contribution due to a layer of adsorbed molecules.

The theory of the ionic double layer is associated with the names of CHAPMAN[7], GOUY[8] and DEBYE and HÜCKEL[9]. Its first main success lay in the elucidation of the osmotic properties of strong electrolytes, and later, the transport properties, for example, conduction and viscosity[10]. More recently it has been applied with equal success to explain many of the classical problems of colloid science which are associated with phase boundaries. It is with these developments that we shall be mainly concerned in the present article. We shall only attempt to cover work done in the last decade, although it will of course be necessary to move outside this range of time on occasions. The next section contains a brief account of the basic theory of the ionic double layer and the general methods available for tackling problems in which it plays an important rôle. The third and main section is a detailed account of the application of the theory to specific problems. Owing to the great extent of the field it is not possible in an article of the present kind to give more than a sketch of the more important investigations; attention will be confined mainly to the physical assumptions and the main conclusions to be drawn from the investigations. We shall pay very little attention to the experimental side and other aspects of electrical phenomena at interfaces. For detailed accounts of these topics the reader may consult recent books by BUTLER[11], MCBAIN[12] and ALEXANDER and JOHNSON[13].

Finally, it may be of interest to make a few observations on the connection of the subject matter of this article with biophysical problems. Practically all the questions we shall discuss are ones usually assigned to colloid chemistry rather than biophysics. Nevertheless, it seems very likely that a close study of these problems will be of increasing value to the biophysicist, as factual knowledge of the complicated processes in living material develops. In the first place certain standard techniques of biophysics, for example the electrophoresis and sedimentation of biological material, involve electrokinetic effects. For a proper understanding of these effects double layer theory is essential. Moreover, many of the fundamental processes observed in living material bear close similarities with important mechanisms found in colloidal systems. For example we may compare mitosis and meiosis with the coagulation of sols and the coiling behaviour of long polymer molecules in electrolytes. It seems highly probable therefore that much of the recent work on colloidal systems will prove fruitful in the more difficult field of biophysics, and that double layer theory will be as important in the latter as it has been found to be in the former.

2. *The general theory of the ionic double-layer*

The type of assembly with which we have to deal consists of solids in contact with liquids or separate liquid phases, the liquids containing

dissolved material. First we may distinguish three main types of system :

(*a*) Systems in thermodynamical equilibrium.

(*b*) Systems not in thermodynamical equilibrium but in a steady state; that is, all relevant physical quantities are independent of the time.

(*c*) Systems not in a steady state and not thermodynamically in equilibrium.

The difficulty of analysis generally increases as we pass from class (*a*) to class (*c*) problems. Many of the chief features of those of classes (*b*) and (*c*) however, can be deduced from models of class (*a*) provided suitable conditions are imposed. For example, the coagulation of colloidal sols is strictly a problem of category (*c*), but the main factors governing the phenomenon can be deduced from a simplified model of class (*a*). Similarly, electrokinetic effects are generally treated as being of type (*b*) although in some cases they should strictly be placed in class (*c*).

Let us first consider problems of type (*a*). The basic method of analysis by means of double-layer theory consists in the calculation of the time average of the electrostatic potential in the neighbourhood of the phase interfaces of the system. From this solution for the potential, the required observed physical properties can be found by various techniques. For example, in the coagulation problem (section 3), we shall show how the free energy of two solid particles immersed in an electrolyte can be obtained from the potential solutions. The variation of the free energy with the separation gives the force between the particles due to electrical effects.

According to the DEBYE-HÜCKEL theory of strong electrolytes, most inorganic compounds and many organic materials are dissociated into free charged ions in solution provided that the dielectric constant of the solvent is sufficiently high. It might be thought, therefore, that when a charged surface is immersed in an electrolyte the ions of opposite sign would move to the surface and neutralise the charge. This is only partially correct, however, since it must be remembered that the ions are not stationary, but executing rapid random Brownian motion. This tends to neutralise any variations in concentration. The net effect therefore is a reduction in the neighbourhood of the charged surface in the average number of ions bearing the charge of the *same* sign as the surface charge, and an increase in the number of ions of opposite sign.

Now let us try and formulate these ideas on a quantitative basis. First consider the case of a solution in thermodynamic equilibrium. If a small element of volume surrounding a fixed point is isolated, we can associate a long time average m_i of the number density for a particular ion type with the small element of volume. In general, m_i will depend on the potential of the ions of type i at the point in question. An exact relation can be written in the form

$$m_i = n_i \exp\left(-v_i/kT\right), \qquad \ldots\ldots (2{\cdot}1)$$

where v_i is a suitably defined *average* potential and n_i is the value of m_i for v_i set equal to zero. An adequate derivation of Eq. (2·1), together with a discussion of the form of averaging required to ascertain v_i is beyond the scope of this article, and for further details the reader is referred to standard texts on statistical mechanics[14]. To make further progress it is now necessary to make some assumption about v_i. It is postulated now that

$$v_i = e\, z_i\, \psi\,, \qquad \ldots\ldots (2{\cdot}2)$$

where $e\, z_i$ is the charge on the ion of type i, and ψ is the time average of the electrical potential at the point in question. Next it is assumed that POISSON'S equation applies to the average potential and ψ average charge density ρ.

$$\nabla^2 \psi = -\frac{4\pi\rho}{\epsilon}\,, \qquad \ldots\ldots (2{\cdot}3)$$

where ϵ is the dielectric constant of the solvent, assumed uniform. But ρ is related to m_i by the equation

$$\rho = e \sum_{i=1}^{s} z_i\, m_i\,, \qquad \ldots\ldots (2{\cdot}4)$$

where the summation is over all the ionic species present. Now combining all the four above equations we finally obtain the following differential equation for the potential.

$$\nabla^2 \psi + \frac{4\pi e}{\epsilon} \sum_{i=1}^{s} n_i\, z_i \exp\left(-ez_i\psi/kT\right) = 0\,. \qquad \ldots\ldots (2{\cdot}5)$$

For a given electrolyte all the quantities in this equation are known except ψ. Hence if the boundary conditions on the potential are known it can in principle be determined completely. From it all the other variables, for example m_i and ρ are known from the above equations. Hence the whole physical problem of finding the average charge distribution in the system is reduced to the mathematical one of solving Eq. (2·5). It must be noticed however that Eq. (2·5) does not follow immediately from Eq. (2·1) which embodies the ordinary postulates of statistical mechanics only; two further additional assumptions, namely those implied in Eqs. (2·2) and (2·3) are involved. Although these are plausible, their truth is by no means intuitively obvious. In fact we can see that Eq. (2·2) may be seriously in error for ions near a charged surface. For, owing to the discrete nature of the ions, if the dielectric constants of solid and liquid differ, the ions will experience image forces in the neighbourhood of the surface. The direction of the force will be independent of the sign of the charge on the ion and so cannot be included in the expression on the right-hand-side of Eq. (2·2).

Several attempts have been made to justify the DEBYE-HÜCKEL procedure, or at any rate to fix limits for its validity (ONSAGER[15], KIRKWOOD[16]). The results are not very helpful however since they indicate that Eq. (2·5) may only be strictly true in the limit of infinite dilution, and not much information is given on the magnitude of the errors involved in its use for finite concentrations. For this reason some writers seem to question the value of the whole theory. But the main purpose of any theory is to explain experimental facts on the basis of a few simple hypotheses. On this criterion the whole body of theory based upon Eq. (2·5) can be looked upon as extremely successful, at any rate for dilute or moderately dilute solutions. It would, of course, be justifiable to discard the theory if another method of equal facility, but without postulates going beyond the basic ones of statistical mechanics, could be devised. As yet, however, no such method or theory is available.

At this point, however, two recent attempts to develop a theory of electrolytes without using the DEBYE-HÜCKEL approximations may be mentioned. The first, due to MAYER[17], is based on the MAYER-MAYER theory of imperfect gases. As early workers found, the ordinary statistical mechanical theory of imperfect gases cannot be applied as one might expect to solutions of ions, since the integrals involved in the partition function, from which physical obervables are derived, diverge in consequence of the slow fall away (as the reciprocal of the distance) of the Coulomb forces. MAYER's analysis is essentially a method of turning this difficulty. The Coulomb force between two ions at distance r is multiplied by a 'convergence factor', $\exp(-\mu r)$, where μ is a small positive constant. The convergence factor ensures that the integrals of the partition function do not diverge and it is ultimately allowed to tend to zero in the final results. Only certain types of the whole gamut of 'cluster integrals' required are evaluated however.

The second is a very interesting analysis due to BERLIN and MONTROLL[18]. This is an attempt to improve an earlier analysis due to KRAMERS[19]. KRAMERS's method is again an ingenious attempt to overcome the difficulty of the divergence of the partition functions. It was possible to show that the DEBYE-HÜCKEL laws were justified in the limit of infinite dilution. But in two respects the theory was very disappointing; with increasing concentration KRAMERS's theory and the experimental results diverged from the DEBYE-HÜCKEL theory in opposite directions. Also the theory could only be applied for concentrations below a certain critical value which turned out to be very low. BERLIN and MONTROLL use KRAMERS's basic method but compute thermodynamic averages over an ensemble which is subjected to a restricting condition. They obtained deviations from the DEBYE-HÜCKEL laws in the same direction as the experimental deviations, but of far too small a magnitude. A serious defect of both the

KRAMERS and BERLIN-MONTROLL methods lies in their restriction to *point* ions, and it is difficult to see how the methods can be modified to take into account short range forces. This is because the whole analysis rests on a mathematical theorem due to KLEIN[20] on homogeneous functions, which is applied to the partition function. Introduction of short range forces destroys the homogeneous nature of the potential energy function and so the theorem cannot then be applied.

Both the theories are of interest for our present purpose in that they help to some extent to justify the DEBYE-HÜCKEL procedure. But both apply only to ordinary assemblies of ions. In view of their intrinsic difficulty the possibility of extending them to the type of problems of interest in colloidal chemistry seems remote.

For an alternative derivation of Eq. (2·5) an interesting paper by COOLIDGE and JUDA[21] may be consulted.

Now let us consider the solution of Eq. (2·5). As it stands this is difficult since the equation is non-linear. DEBYE and HÜCKEL made the further approximation, known by their names, that

$$e\, z_i\, \psi \ll k\, T\,. \qquad \ldots\ldots (2{\cdot}6)$$

Since we may take the solution to be electrically neutral if ψ is zero everywhere, so that

$$\sum_{i=1}^{s} n_i\, z_i = 0\,, \qquad \ldots\ldots (2{\cdot}7)$$

Eq. (2·5) becomes, on expanding the exponential and ignoring all terms of powers of ψ higher than the first, in accordance with condition (2·6)

$$\nabla^2 \psi - \kappa^2 \psi = 0\,, \qquad \ldots\ldots (2{\cdot}8)$$

where

$$\kappa^2 = 4\pi\, e^2 \sum_{i=1}^{s} n_i\, z_i^2 / \epsilon\, k\, T\,. \qquad \ldots\ldots (2{\cdot}9)$$

Eq. (2·8) is now linear in ψ and the solutions in most cases of practical interest are not difficult to obtain. For details the reader may refer to Chapters 2 and 4 of a book by VERWEY and OVERBEEK[22]. To take two very simple cases, if the interface of Figure 1 is a plane then the potential in the electrolyte is

$$\psi = \psi_0\, e^{-\kappa x}\,, \qquad \ldots\ldots (2{\cdot}10)$$

where x denotes distance measured normal to the solid-liquid boundary into the liquid. From Eq. (2·10) it will be seen that ψ falls to a small fraction of ψ_0 in a distance equal to $1/\kappa$. For this reason the reciprocal of κ

is sometimes known as the *double layer thickness*. If the boundary is a sphere of radius a, the potential is

$$\psi = \psi_0 \frac{a}{r} e^{\kappa(a-r)}, \qquad \ldots . (2{\cdot}11)$$

where r is the distance from the centre of the sphere.

One solution of considerable importance which is rather difficult however is that for two charged spheres separated by a distance comparable with their radii and with the double layer thickness. No simple analytical solution is possible for this case and various series solutions are required. For details the reader may refer to papers by LEVINE[23], and LEVINE and DUBE[24].

Unfortunately, condition (2·6) is rather a severe one since it implies that at room temperatures ψ must be small compared with 25 millivolts. Experimental work suggests that the potential ψ_0 of Figure 1 may sometimes reach values of the order of 150 millivolts. Evidently a solution based on Eq. (2·8) might be expected to give results very much in error with systems carrying potentials of this order, and the complete Eq. (2·5) must be used. The solution of this equation is far more difficult than that of Eq. (2·8) and the only straightforward case is that of a plane boundary with a simple binary electrolyte. A fairly general method of solving Eq. (2·5) involving expansions of the potential in power series in terms of the charges on the boundaries has been given by BOOTH[25].

A further complication may be of importance in the higher range of potentials. In Eq. (2·3) the constancy of the dielectric constant ϵ throughout the electrolyte has been assumed. It can easily be shown however that in the diffuse ionic double layer very intense electrical fields may develop provided that the boundary potential ψ_0 is sufficiently high. At these fields the dielectric may saturate and so the effective value of the dielectric constant ϵ may be much less than its usual value. This question has been examined by BOCKRIS *et al.*[26]. Even for plane boundaries it is now necessary to find the potential by numerical methods. BOCKRIS found that the dielectric saturation may be of considerable importance for aqueous electrolytes; but for the quantitative aspect of saturation he used an early theory of DEBYE which overestimates the reduction of the dielectric constant. Recent work by CHESTER and BOOTH[27] suggests that the saturation effect may not after all be of very great importance in double layer theory.

Now let us examine the analysis of systems of categories (*b*) and (*c*). Eqs. (2·3) and (2·4) are still valid, but Eq. (2·1) only holds for systems in thermodynamic equilibrium and so can no longer be applied. In its place it is now necessary to introduce the equations of motion and of continuity for the ions. Since the motion of the ions depends upon that of the solvent it usually also becomes necessary to examine the motion of the electrolyte

too. In view of these complications it is not surprising that further approximations are generally introduced.

With this we shall conclude our general account of the basic theory of the ionic double layer and we shall now show how it has been applied to specific problems. First, problems involving systems in equilibrium, that is of type (*a*), will be considered and later type (*b*) and (*c*) problems will be examined.

APPLICATIONS OF THE THEORY OF THE IONIC DOUBLE-LAYER

3. *The stability of lyophobic colloids*

The most important properties of colloids that we shall discuss are their stability and the conditions required for coagulation. First however it will be necessary to give a brief summary of the physical properties which the theory has been called upon to explain. To begin with it is necessary to explain the meaning of the term colloidal sol. There is no fundamental physical difference between sols and ordinary suspensions. Both consist of finely dispersed solid particles suspended in a liquid. The distinction lies in the *size* of the particles. Roughly speaking, a suspension will settle in a time up to a few hours, whereas a sol may take days, or may even last indefinitely. The critical size separating the two is about 10^{-4} cms when the suspending medium is water. In theory sols could have any particle size from about 10^{-3} cms down to atomic dimensions. Sols with a particle size of less than 10^{-6} cms, which are sometimes known as *amicronic sols* or *molecular dispersions*, are however very unstable and difficult to prepare.

Next we must distinguish between *lyophobic* and *lyophilic* sols. As the names imply, in the first the particles have no affinity for the suspending medium, but in the second they have considerable affinity. The nature of the particles forming lyophobic sols is the same as that of the material in the macroscopic state. Lyophilic sols on the other hand owe many of their characteristic properties to strong chemical binding between particle molecules and neighbouring solvent molecules. The theory of lyophilic sols is much less developed than that of lyophobic, and in what follows we shall be dealing with the latter. The process of formation of the sol from the finely divided solid and the pure liquid is known as *peptisation*. In a few instances the sol may be prepared simply by mixing solid and liquid vigorously. But in the majority of cases it is necessary for the liquid to contain suitable small quantities of certain ions for successful peptisation to occur. Thus oxides and metals are peptized by hydrogen and hydroxyl ions; silver halide sols require silver ions or the corresponding halide ion.

Under suitable conditions the particles forming a sol, instead of remaining freely suspended, may tend to crowd together and form large

loose clusters. The whole of the solid matter eventually precipitates. This effect, known as *flocculation* or *coagulation*, can be brought about either by removing the peptizing ions from the liquid, a procedure known as depeptization, or by adding sufficient quantities of an indifferent electrolyte; that is, an electrolyte which may be unconnected with or not directly affect the peptizing ions. The conditions for coagulation by the second method have been extensively studied and the main results are embodied in the well-known SCHÜLZE-HARDY rules:

(*a*) For a given sol, the concentration of indifferent electrolyte at which coagulation occurs is nearly independent of the nature of the ions present in the indifferent electrolyte, bearing charge of the same sign as the charge on the particles.

(*b*) The limiting concentration for coagulation depends only on the valency of the ions bearing charges of the opposite sign from the charge on the particles. For *simple* electrolytes, that is those containing a single salt, the limiting concentration is inversely proportional to the *sixth* power of the valency.

Exceptions to these rules occur, especially with large organic ions. Materials both organic and inorganic which do not ionise have little effect on colloidal stability, provided of course that no chemical reaction occurs.

Instead of flocculating, lyophobic sols may solidify as a whole on addition of electrolyte, an effect known as gelation. Although superficially the effect seems very different from coagulation, the two are evidently closely related since the SCHÜLZE-HARDY rules often still apply to gelation. In the solid form the system is known as a *gel*.

Now let us see how the above experimental facts have been largely explained by the theory of the ionic double-layer. Satisfactory explanations of the apparent stability of lyophobic sols and their behaviour with electrolytes as summarized in the SCHÜLZE-HARDY rules are required.

The first point to notice is that lyophobic sols are essentially unstable systems. Since the particles forming the sol are very fine, the surface area of the solid-liquid interface is very large. Consequently the free energy of a sol is much greater than the system containing the same material in two ordinary distinct phases. Thus an ordinary sol is in fact always coagulating, but at a rate which is so extremely small that it can be regarded as perfectly stable for ordinary purposes. Since two particles in a coalesced state have less free energy than when they are separated it is necessary to account for the strong potential barrier between them which makes coalescence so rare. An obvious answer to this question is that the repulsion is due simply to the charges carried by the particles. The coagulation by electrolytes may then be explained very simply as due to adsorption of oppositely charged ions by the particles. This theory, due to FREUNDLICH[28], was accepted for many years.

There are two serious objections to it, however. It has proved impossible to demonstrate conclusively that the charge is in fact reduced by addition of electrolyte, and in some cases it seems likely that the charge is actually increased. Secondly, it is extremely difficult to see how any conceivable explanation of the SCHÜLZE-HARDY rules could be given on this theory. For it would be expected that the coagulating efficiency of an electrolyte would depend very markedly on the specific chemical nature of the ions present in the electrolyte, not simply on their valency.

The present view is that colloidal stability is to be explained in terms of the interplay of VAN DER WAALS' and electrical forces between the particles, the electrical forces being strongly influenced by the presence of the ionic double-layer surrounding the particle boundaries. KALLMANN and WILLSTÄTTER[29] seem to have been the first to put forward this idea. The physical basis of the theory can be understood from the following very simple model. We take two massive point particles, each carrying a charge e and immerse them in an infinite liquid of dielectric constant ϵ, containing no charged ions. Then the mutual potential energy V, as a function of the separation r, is given by

$$V = \frac{e^2}{\epsilon r} - \frac{a}{r^6} . \qquad \ldots . (3{\cdot}1)$$

The first term is the ordinary electrical one and the second the VAN DER WAALS' interaction; a is a constant. According to LONDON's theory[30], the second term varies as the inverse sixth power of the distance. The particular shape of the V-r relation depends upon the values of the constants, e, ϵ and a. But it is important to realise that whatever values these constants may have it is always of the same *form*. V will always be negative for r sufficiently small, but positive for r sufficiently large. Thus, provided that r is greater than the value corresponding to the maximum of V, that is for $r = (6\ a\ \epsilon/e^2)^{\frac{1}{4}}$, the particles will repel each other. Now suppose material which can ionise is added to the liquid. The second term of Eq. (3·1) will be unchanged but the first will be reduced more and more as r increases, since it must now be multiplied by the factor $\exp(-\kappa r)$, where κ is defined by Eq. (2·9). This result follows easily from Eq. (2·11). It is now easy to show, using this modified expression for V, that by making κ sufficiently large, V can be made to be negative for all values of r; physically this means that the particles will now attract each other for all r. Since κ is proportional to the square root of the concentration of electrolyte, it follows that the two particles can be made to coalesce simply by adding sufficient electrolyte. Further, since κ depends only on the concentration of the ions and their valency, not on their specific chemical nature, the limiting concentration of electrolyte for coagulation will depend only on the valencies of the ions. We see therefore that the model gives a

simple qualitative account of some of the observed facts concerning the coagulation of sols. But we do not obtain the second SCHULZE-HARDY rule since it transpires that the coagulating concentration would be proportional to the inverse *square* of the valency of the counter-ions, not the inverse sixth power.

Thus we see that although our simple model reproduces several of the important qualitative features of colloidal stability and coagulation, it is, as one might expect, quite inadequate to describe real systems in detail. To analyse these it will be necessary to generalize the model in the following ways :

1. In a real sol we have particles of finite extent not point masses. This will have two effects :

(*a*) The VAN DER WAALS' attraction between a pair will not necessarily be given by the simple inverse sixth power law. The force will depend on the size and shape of the particles.

(*b*) The simple solution used for the electrostatic interation will no longer obtain. It will be necessary to obtain the appropriate solutions for the electrostatic potential distribution in the system, as explained in Section 2, and from these calculate the electrostatic force between the sol particles.

2. In the model we have considered a single *pair* of particles in an *infinite* liquid; ordinary sols consist of a *distribution* containing a large number of particles enclosed in a *finite* volume of liquid.

3. It has been assumed implicitly in the model that the charges carried by the particles are fixed entities like their masses. In any real sol this state of affairs will be the exception rather than the rule. The charge on the particles is one effect of some form of chemical equilibrium between the electrolyte and the solid surface; for example, it may be due to preferential adsorption of certain ions. Hence the surface charge density will depend on local conditions in the liquid. It follows that :

(*a*) The surface charge density will, in general, be a function of the distance between the particles;

(*b*) The total quantities of ions present in the electrolyte may also alter with the distance.

4. To calculate the electrical force the DEBYE-HÜCKEL approximation has been used. This implies that $e\,\psi/k\,T$ is small compared with unity everywhere. In colloidal systems this condition is certainly not always fulfilled. Hence it is necessary to work with the more general POISSON-BOLTZMANN equation, Eq. (2·5) rather than with Eq. (2·8).

5. It is assumed in the model that the dielectric constant is strictly a constant. As explained in Section 2, owing to the strong field in the ionic double layer, ϵ may be reduced by saturation.

6. The particles of a sol are not fixed but execute Brownian motion.

This must be taken into account in considering stability.

7. The discrete structure of the ions forming the electrolyte is ignored.

This list is clearly a very formidable one. It would be too much to hope that all the difficulties embodied in it could be resolved in a single ' magnum opus.' But recent theoretical work can be regarded as successive attempts to improve our simple model in one or more respects. To understand the significance and the mutual relationships between the numerous papers it is convenient to examine them with reference to the above list. The authors, of course, did not have an explicit programme of this type in mind. In what follows, for brevity, we shall refer to the various generalizations in the above list by the symbol G.

The first important advance was due to HAMAKER[31] and DE BOER[32] who gave the relevant calculations of the VAN DER WAALS' attraction between *extended* particles of suitable simple shapes, so partially solving problem G1(a). DE BOER calculated the attraction between two infinite solids with parallel plane faces separated by a distance d, the region between the plates containing no matter. For the potential he found

$$V_w = -\frac{A}{12\pi d^2}, \qquad \ldots\ldots (3{\cdot}2)$$

where

$$A = \pi^2 \sum_s q_s^2 \lambda_s . \qquad \ldots\ldots (3{\cdot}3)$$

q_s is the number of atoms in unit volume of the material of species s. λ_s is the fundamental VAN DER WAALS' constant, defined by the attraction between a pair of atoms. Thus if r is the distance apart, the attractive potential is $-\lambda_s/r^6$. The summation in Eq. (3·3) is over all the atomic species present and formula (3·2) presupposes uniform distribution of the various species. The calculation of λ_s is a matter for quantum mechanics. It is only possible to get approximate formulae, but the most useful relation is due to SLATER and KIRKWOOD[33]

$$\lambda_s = 11{\cdot}3 \times 10^{-24} n_s^{\frac{1}{2}} \alpha_s^{\frac{3}{2}} \qquad \ldots\ldots (3{\cdot}4)$$

where n_s is the number of electrons in the outer-most shell of the atom of type s; α_s is its polarisability. The LONDON theory of VAN DER WAALS' forces shows that the forces are simply additive as in gravitational attraction. Hence Eq. (3·2) is easily derived by a straightforward integration. VERWEY and OVERBEEK[34] have generalized DE BOER's theory to give the attraction between two infinite plates of equal finite thickness with parallel surfaces.

Although DE BOER's calculation is valuable for calculations on sols, his paper was not concerned with colloidal solutions but the bonding forces in molecules. HAMAKER[31] gave results of more direct significance. In

colloidal solutions we are not dealing with particles in vacuo but immersed in a second medium, and each particle replaces an equal volume of the medium. Thus, if A_p is the constant for the interaction between the solid atoms, A_s for the liquid atoms, and A_{sp} for the interaction between solid and liquid, then in Eq. (3·2) if we desire to apply the formula to sol particles, we must use, instead of A_p simply, the constant A defined by

$$A = A_s + A_p - 2\,A_{sp}\,. \qquad \ldots\ldots (3{\cdot}5)$$

Since all the constants A_s, A_p and A_{sp} are necessarily positive, it is not immediately obvious that A is also always necessarily positive. In other words, although two particles in vacuo attract one another, when they are immersed in a medium, owing to the displacement of part of the medium, they may now actually repel one another. HAMAKER was able to prove quite generally, however, that A defined by Eq. (3·5) is *always* positive. Hence, VAN DER WAALS' forces always lead to *attraction.*

HAMAKER, in the same paper, also calculated the attractive potential between two spheres. For equal spheres of radius a with their centres d apart, he found

$$V_w = -\frac{A}{6}\left[\frac{2\,a^2}{d^2-4a^2} + \frac{2\,a^2}{d^2} + \log_e \frac{d^2-4a^2}{d^2}\right]. \ldots\ldots (3{\cdot}6)$$

In three other papers HAMAKER[35] investigated the interaction of a pair of particles. Although adequate expressions for the VAN DER WAALS' interaction energy were used, very rough approximations, only slightly more exact than those of our simple model, were employed for the double layer interaction energy. Consequently, we shall not review this work in detail since it has been superseded by later calculations, although it might be noted that HAMAKER was able to explain various effects qualitatively.

Before proceeding with the historical development it may here be pointed out that for *large* distances LONDON's formula requires correction for retardation effects. These arise because of the finite time of travel of electromagnetic disturbances; in LONDON's theory it is assumed that the velocity of propagation is infinite. The question has been studied by CASIMER and POLDER[36]. An exact analysis is out of the question. They showed, however, that the energy (LONDON or VAN DER WAALS') between a neutral atom and a perfectly conducting wall varies as r^{-4} for *large* distances, whereas the LONDON theory gives a variation as r^{-3}, r being the distance of the atom from the wall. For two *atoms* the VAN DER WAALS' force for large distances is given very nearly by

$$V_w = -\frac{23\,\hbar\,c\,\alpha_A\,\alpha_B}{4\,\pi\,r^7}\,, \qquad \ldots\ldots (3{\cdot}7)$$

where α_A and α_B are the polarisabilities and r the separation, so that V falls away as r^{-7} instead of r^{-6} as in the LONDON theory.

Meanwhile, other workers made progress in the evaluation of the electrical interaction energy for extended solid surfaces (G1b). DERJAGUIN[37] obtained the repulsive force between two infinite uniformly charged plates in an electrolyte. If x denotes distance measured perpendicular to either plate, p is the hydrostatic pressure and ψ the potential, then for equilibrium we must have

$$\frac{dp}{dx} + \rho \frac{d\psi}{dx} = 0 , \qquad \ldots . (3{\cdot}8)$$

where ρ is the charge density in the double layer. Substituting for ρ by POISSON's equation we have

$$\frac{dp}{dx} - \frac{\epsilon}{4\pi} \frac{d^2\psi}{dx^2} \frac{d\psi}{dx} = 0 . \qquad \ldots . (3{\cdot}9)$$

This is a simple equation relating p and $d\psi/dx$. From it the repulsive force per unit area is easily found. Using the solution for the potential involving the DEBYE-HÜCKEL approximation we have for this force

$$f(h) = \frac{\epsilon \kappa^2 \psi_0^2}{8\pi \cosh \kappa h/2} , \qquad \ldots . (3{\cdot}10)$$

where ψ_0 is the potential at the plates and h their separation.

DERJAGUIN[38,39] also devised a simple ingenious method of calculating the repulsion between two spherical particles. The method is approximate, however, and only applies when the spheres are close together and the double layer thickness is small compared with the radius so that the main contribution to the repulsion arises from small areas of the surface enclosing the line of centres.

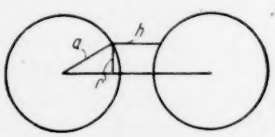

Figure 2.

Suppose in Figure 2 the surfaces of the spheres are divided into a series of rings of radii r, the centre of each ring being on the line of centres. Then DERJAGUIN suggests that the total repulsion may be written

$$g(h_0) = 2\pi \int_0^a f(h)\, r\, dr , \qquad \ldots . (3{\cdot}11)$$

where $f(h)$ is given by Eq. (3·10) and h_0 is the minimum value of h. This formula is clearly approximate but it should be fairly adequate provided that the main contribution to the integral arises from the *parallel* parts of the two surfaces, that is for r small. Hence the restriction on the

thickness of the double layer. In view of this restriction we can replace the upper limit of the integral in Eq. (3·11) by infinity. Also we have

$$r\,dr = \frac{a\,dh}{2}\left(1 - \frac{h^2}{a^2}\right)^{\frac{1}{2}} . \qquad \ldots . (3{\cdot}12)$$

Since contributions to the integral when h is not small compared with the radius may be ignored, $(1 - h^2/a^2)^{\frac{1}{2}}$ can be replaced by unity, and Eq. (3·11) becomes

$$g(h_0) = \pi a \int_{h_0}^{\infty} f(h)\,dh . \qquad \ldots . (3{\cdot}13)$$

Substituting Eq. (3·10) for $f(h)$ we find

$$g(h_0) = \frac{\epsilon \kappa a \psi_0^2}{4}\left[1 - \tanh \frac{\kappa h_0}{2}\right]. \qquad \ldots . (3{\cdot}14)$$

Although the restriction on the double layer thickness limits the usefulness of the method, nevertheless it is of considerable interest since it can evidently be extended to give the electrical forces between particles of less simple shapes; exact calculations in these cases would be extremely difficult or intractable.

LANGMUIR[40] generalized Eq. (3·10) by using the complete POISSON-BOLTZMANN equation, Eq. (2·5). The only change in the calculation is that the different solutions for ψ must be substituted in Eq. (3·9). For a simple binary electrolyte LANGMUIR found

$$f = 2\,n\,k\,T(\cosh e\,\psi_m/k\,T - 1) \qquad \ldots . (3{\cdot}15)$$

where ψ_m denotes the potential at the point midway between the plates. In Eqs. (3·10) and (3·15) it has been assumed that ψ is zero at infinity.

The next contribution of importance was due to CORKILL and ROSENHEAD[41]. These writers made the observation that in many colloidal systems the solid particles seem to take the form of plates. They therefore endeavoured to calculate the force between two plane charged parallel surfaces in an electrolyte, without using the DEBYE-HÜCKEL approximation; that is, they endeavoured to solve problem G4 for this model. In the first part of the paper correct solutions for the potential are developed. In the second part the electrical force between the plates is calculated. They realised that the charge may vary with the separation, as explained in G3a above; since they had no independent guide to show how the charge did vary in practice they calculated the force for three special cases:

1. When the potential difference between the surface of the plates and the midpoint between them remains independent of the separation.

2. The potential at the mid-point remains independent of the separation.
3. The surface charge density is independent of the separation.

In cases (2) and (3) they found an **attraction** between the plates, but in case (1) a repulsion. They did not introduce VAN DER WAALS' forces or discuss stability in detail. But the result of the calculation indicated that possibility (1) alone could account for stability.

The attraction in cases (2) and (3) above seems very curious since in our model it is clear that the double-layer reduces the ordinary electrostatic repulsion, but it is difficult to see how it can change the repulsion to attraction. Furthermore, CORKILL and ROSENHEAD'S results are in disagreement with LANGMUIR'S. Eq. (3·15) was calculated without any special assumptions being made as to how the charge alters with the plate separation.

The discrepancy is due to fundamental mistakes in CORKILL and ROSENHEAD'S method of calculating the force. They first calculated the electrical energy from the formula

$$W = \frac{\epsilon}{8\pi} \int_0^h \left(\frac{d\psi}{dx}\right)^2 dx \qquad \ldots\ldots (3{\cdot}16)$$

where h is the distance between the plates, and then investigated the gradient of W with respect to h. But the repulsive force is the gradient of the HELMHOLTZ free energy, usually denoted by the symbol F, with respect to the separation; hence it is necessary to ascertain whether in fact F and W can be identified. It seems very plausible that they can be since in the case of ordinary dielectrics W does represent the free energy (FRÖHLICH[42]). But for the present problem W is not the same as F and the two functions exhibit entirely different behaviour as functions of h. There are two reasons for this; even in case (3) when the charge on the plates is taken as fixed, the ionic charges are not stationary but moving around rapidly due to their Brownian motion. In cases (1) and (2) a further error occurs since energy changes accompany the removal or deposition of ions on the surfaces. It is of interest to notice that in the ordinary DEBYE-HÜCKEL theory of electrolytes the configurational electrical energy and the free energy are not identical, the former being one and a half times the latter. For further details the reader is referred to VERWEY and OVERBEEK'S book[43].

We now come to a long series of papers by LEVINE[44,45] and LEVINE and DUBE[46]. In these papers the authors attempt to find the electrostatic interaction between two *spherical* particles using the approximate form of the POISSON-BOLTZMANN equation. They therefore tackled the same problem as that solved by DERJAGUIN in 1935, with the difference that the charged solid surfaces were spheres instead of planes. As we have already

explained in Section 2, the solutions of the potential equation for this arrangement are rather troublesome and intricate analysis was required. The results gave attraction at large separations with repulsion at small separations. At first sight this might appear to indicate that the phenomenon of stability could be explained purely by means of the electrical interaction without introducing VAN DER WAALS' forces at all. But, as HAMAKER[47] pointed out, the calculations gave minima in the energy curves at distances between the centres which were not very much greater than the diameter. Introduction of VAN DER WAALS' forces could only reduce the separation for minimum energy. Hence the calculated energy curves indicated that a sol could never be stable.

It will not be necessary, however, to discuss these papers in detail since the results were spurious. Although the solutions given for the potential distribution were perfectly correct, the authors made precisely the same error as CORKILL and ROSENHEAD in their calculation of the interaction force. They derived this from the configurational energy (Eq. (3·16)) instead of from the free energy.

DERJAGUIN[48] seems to have been the first to notice the error in the method of calculating the force from the configurational energy rather than the free energy. He showed that the free energy for small potentials can be written under fairly general conditions in the form

$$F = \frac{\epsilon}{8\pi} \int\int\int \left[\left(\frac{\partial \psi}{\partial x}\right)^2 + \left(\frac{\partial \psi}{\partial y}\right)^2 + \left(\frac{\partial \psi}{\partial z}\right)^2 + \kappa^2 \psi^2 \right] dx\, dx\, dz\,, \quad (3{\cdot}17)$$

where the volume integral extends through the electrolyte. Using some of the approximate solutions for ψ mentioned in Section 2, he obtained various simple formulae for the electrostatic force. We shall not discuss these in any detail, however, since he did not introduce VAN DER WAALS' forces, and the formulae were only valid for sufficiently small potentials for condition (2·6) to apply.

We now come to the work of VERWEY and OVERBEEK[22] which is the most important contribution in the whole field. It will be clear to the reader that until 1940, although many authors had obtained valuable results, the central problem remained quite unsolved. Moreover, some of the calculations gave answers so much at variance with the experimental facts that it seemed possible that the whole basis of the theory might be wrong. VERWEY and OVERBEEK made two major advances. First, they devised sound and workable formal methods of calculating the electrical interaction, so solving completely problems G1(b) and 4. Secondly, they carried out a large amount of detailed calculation which gave a very good account of the data on stability, and in particular they obtained a theoretical proof of the SCHÜLZE-HARDY rule. Subsequent work, mainly by LEVINE, has shown that their treatment may be refined in various ways, but this

has not altered the fundamentals. Owing to the difficulties of publication in wartime, the results of the work, which was done in the years 1940-1944 in Holland, were not published in the ordinary way but in book form. Shorter reviews[49] have since appeared, however.

In their first method of calculating the free energy VERWEY and OVERBEEK used the following process. The solid surfaces are brought into contact with the electrolyte and the double-layer is built up isothermally by allowing infinitesimal quantities of ions to be transported to the surface in steps. After each small transfer the system is allowed to come to equilibrium isothermally before the next transfer is effected. When all the charge required to give the surfaces the potential obtaining under equilibrium conditions has been conveyed, and rearrangement is complete, the total work which has been done represents the free energy. This work consists of three terms :

1. The ions on the surface have a different chemical potential from those in solution far away from the surfaces. Otherwise no double-layer could be formed. Let $\Delta\mu_i\ (s_i(P))$ denote this difference for the ions of type i at a certain point on the surface S where the surface density (that is, number of ions per unit area) is $s_i\ (P)$. Then, in the charging process an amount of work equal to

$$F_c = \sum_{i=1}^{s} \int_S d\,S \int_0^{s_{io}(P)} \triangle\ \mu_i\,(s_i\,(P))\,d\,s_i\,(P)\ , \qquad \ldots . (3{\cdot}18)$$

will have to be done. The summation is over all the ionic species present. The first integral is taken over the whole of the solid-liquid interface S. $s_{io}\,(P)$ is the *final* value of the surface density at P. VERWEY and OVERBEEK call F_c the *chemical* term in the free energy.

2. Work will be required to convey ions from the bulk of the solution at zero potential to the surface S at a different potential. If this contribution is written as F_e we have

$$F_e = \int_S d\,S \int_0^{\sigma_0(P)} \psi\,(\sigma,\,P)\,d\,\sigma\,(P). \qquad \ldots . (3{\cdot}19)$$

The first integral is again taken over S. $\sigma_0\,(P)$ is the charge density at P on the surface when the charging process is complete, whereas $\psi\,(\sigma,\,P)$ is the potential at the surface when the charge density is σ.

3. Finally there will be a contribution, F_t, due to the rearrangement of the ionic charges in the solution.

VERWEY and OVERBEEK now make two simplifying assumptions :

(*a*) $\Delta\ \mu_i$ is independent of s_i. This is not stated explicitly, but is

implicitly assumed in their method. It follows that we can write

$$\triangle \mu_i = - e z_i \psi_0 (P) , \qquad \ldots . (3{\cdot}20)$$

where z_i is the valency of the ions of type i and ψ_0 the final potential of the surface at P. Hence

$$\sum_{i=1}^{s} \int_0^{s_{i0}} \triangle \mu_i \, d s_i = - \sum_{i=1}^{s} e z_i s_{i0} \psi_0 (P) = - \sigma_0 (P) \psi_0 (P) . \quad (3{\cdot}21)$$

(*b*) They point out that there is no major contribution to F_t from the double layer region. The gain in work arising from the increase of ions of opposite sign from those on the surfaces is balanced by their loss of entropy. There will be a contribution to F_t however from the bulk of the solution; this contribution is neglected.

For the total free energy we now have from Eqs. (3·18), (3·19) and (3·21)

$$F = - \int_S \mathrm{d} S \int_0^{\psi_0 (P)} \sigma (P) \, d \psi (\sigma, P) . \qquad \ldots . (3{\cdot}22)$$

When the DEBYE-HÜCKEL approximation can be applied F is easily calculated from this formula. But for higher potentials it was found that the relation was inconvenient to use. Consequently another method of calculating F was devised. This was based on the so-called charging process originally used by DEBYE and HÜCKEL to find the free energy of solutions of strong electrolytes. They found that F could be expressed in the form

$$F = \int_0^1 \frac{d \lambda}{\lambda} \iiint \psi (\lambda, P) \, \rho (\lambda, P) \, dx \, dy \, dz \ldots . (3{\cdot}23)$$

The second integral is a three-fold integration throughout the solution $\psi (\lambda, P)$ and $\rho (\lambda, P)$ denote the potential and charge density respectively at the point P for an equilibrium distribution in which all the charges in the system, including those on the surfaces, have only a fraction λ of their usual values.

At first sight it is by no means obvious that the expressions on the right-hand sides of Eqs. (3·22) and (3·23) are identical. A proof that in fact they are was given by CASIMIR[50]; we shall not go into this since it is merely a question of mathematical manipulation.

Now let us turn to the less abstract part of VERWEY and OVERBEEK's work. They examined with a profusion of numerical detail the interaction of plane parallel plates and spheres. First, we may consider the work on

plates. The important simplification that edge effects were not of importance was made at the outset. From Eq. (3·23) an exact expression for the free energy was found. By differentiating this with respect to the plate separation, assuming ψ_0 remained constant, it was verified that LANGMUIR's formula for the repulsion (Eq. (3·15)) was obtained. This might seem to imply that VERWEY and OVERBEEK made little advance on LANGMUIR's work. But for computational purposes the formula for the repulsive *force* is much less useful than that for the free *energy*. Combining the electrical interaction energy with the VAN DER WAALS' energy given by Eq. (3·2) they plotted curves of the total interaction energy against the plate separation. For sufficiently large or sufficiently small values of the separation d, the total potential V is always negative, since in both cases the VAN DER WAALS' attraction predominates. For large d however, the curves approach the axis $V = 0$ asymptotically.

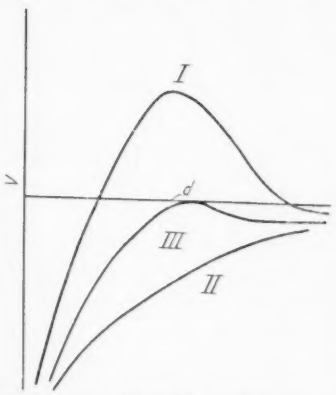

Figure 3. Potential energy as a function of separation for two colloidal particles in an electrolyte.

In the intermediate range of d, two types of potential curve are possible; in curves like I, V has a range of *positive* values, whereas in curves like II, V is always negative. Hence in case II the plates will always attract, but in case I there will be repulsion succeeded by a slight attraction as d is increased beyond the maximum point of the curve. VERWEY and OVERBEEK identified states represented by curves like I with *stable* sols, and states like II by *unstable* sols. Further, they assumed that the transition state between the two cases was determined by the curve III in which the maximum just coincides with the d axis. In doing this they implicitly made three further simplifying assumptions. First, the model is of infinite parallel planes and is only satisfactory provided end effects are not important. It is reasonable to assume that this is justified provided that the dimensions of the particles are large compared with the separation in the region of interest. Examination of the computed curves shows that the maxima occurs at values roughly of the order of the double layer thickness. Hence the dimensions of the plates in the directions perpendicular to their common normal should be large compared with the double layer thickness. Secondly, the Brownian motion of the particles is ignored in the same way as we ignored it earlier in the simple point particle model. This means that the theory may not be good for very fine sols. VERWEY and OVERBEEK show by very simple numerical arguments that for particles

greater than 10^{-6} cm, Brownian motion has little effect, but for particles less than this the critical curve for stability may have to be drawn considerably above curve III in Figure 3. Finally, the very shallow *minimum* in curve III beyond the maximum is ignored.

The change from stable states represented by curves I to unstable ones represented by curves II can be brought about either by reducing the surface potential ψ_0, or increasing the concentration of the electrolyte. Physically, the first corresponds to depeptization, the second to flocculation by electrolytes. For a given surface potential VERWEY and OVERBEEK gave the term 'flocculating concentration' to the concentration giving the limiting curve III. A very good account of the experimental results, including a simple proof of the SCHÜLZE-HARDY rule, could be given by assigning to the constant A in Eq. (3·2) the value $2{\cdot}0 \times 10^{-12}$; this is just the order of magnitude to be expected from the SLATER-KIRKWOOD formula (Eq. (3·4)).

Here it is interesting to note that OVERBEEK and SPARNAAY[51] in a provisional communication, have reported the detection and measurement of VAN DER WAALS' forces between flat plates. Two highly polished glass plates were arranged in vacuo with a very small separation between their parallel faces. Estimates of parallelism and distance were obtained by NEWTON'S rings in white light. The value for the constant A appeared to be of the order 10^{-11}; that is, somewhat higher than the value required for the sol measurements. This may be due to surface roughness.

VERWEY and OVERBEEK also made some calculations for spheres. Owing to the difficulty of solving the POISSON-BOLTZMANN equation for this arrangement they were not able to get completely general expressions for the electrical free energy. They first improved DERJAGUIN'S calculation by using formula (3·15) instead of Eq. (3·10) in Eq. (3·11), and found the total force by numerical integration. From the limitations imposed by the approximations this result could only be valid for κa large, where a is the radius of the spheres. The other case worked out in detail was for κa small, using LEVINE and DUBE'S solutions for the potential. Since these solutions derive from the approximate DEBYE-HÜCKEL equation, Eq. (2·8), it follows that the whole development is only valid provided $e\,\psi_0/k\,T$ is small compared with unity. The curves for the interaction energy as a function of the separation of the spheres had the same general characteristics as the corresponding ones for flat plates, but with differences in detail.

The development of the theory since VERWEY and OVERBEEK'S work is mainly due to LEVINE. In a long series of papers LEVINE has introduced many refinements in, and extended the earlier calculations. The papers are rather intricate mathematically and introduce many subtle points. Consequently in this article we shall not go into many details but merely indicate the broad sweep.

The first paper[52] is mainly concerned with the so-called 'chemical term,' Eq. (3·18) in the VERWEY-OVERBEEK treatment. LEVINE points out that in the derivation of expression (3·18) for F_c it is assumed that the chemical potential of the adsorbed ions is independent of both the surface density of the separate ions and the plate separation. Since we are ultimately concerned with the dependence of the free energy on the plate separation, it is clearly preferable to formulate the theory in a more general way, allowing for possible variations. In fact, instead of *assuming* that the potential of the plates is independent of their separation it should be possible to calculate this potential from the theory, just as the force is derived. LEVINE assumes an expression for the free energy of the adsorbed ions in the uncharged state. Using a very general theorem of statistical mechanics[53], an expression for the free energy when they are charged is derived. The theory is applied to infinite parallel plates with small surface potentials. The result was surprising; for large separations the electrical interaction was attractive although for very small separations it was repulsive. Hence there appeared to be a serious discrepancy with VERWEY and OVERBEEK'S work.

OVERBEEK[54] pointed out a serious anomaly in LEVINE'S analysis. Under the condition of fixed density of adsorbed ions, the surface potential would tend to infinity as the charges on the ions became zero in the hypothetical charging process; a possible way out of the difficulty was suggested.

LEVINE[55], in a short discussion, admitted the difficulty but rejected OVERBEEK'S solution. The matter was cleared up on LEVINE'S second paper of his series[56]. In a re-examination of the basis of the theory it was shown that the results could be obtained from a generalized form of the LIPPMANN equation. It was shown that the paradox pointed out by OVERBEEK was due to neglect of edge effects. In consequence, the electrical attraction was erroneous; if the finite size of the plates is taken into account we always get repulsion.

A further examination of the theory is given in the third paper of the series[57]. The main discussion turns on the question of the equivalence of the two fundamental methods of calculating the electrical free energy. The author examines CASIMIR'S equivalence theorem and, besides pointing out a number of minor errors, gives a more generalized proof. Paper IV[58] contains a further extension of VERWEY and OVERBEEK'S method.

The fifth paper[59] deals effectively with the term F_t in the electrical free energy. It will be remembered that in VERWEY and OVERBEEK'S method this contribution was regarded as a second order effect and dropped. LEVINE shows that it gives an extra contribution to the repulsion of order d^{-6} for spheres, d^{-5} for infinite parallel cylinders, and d^{-4} for infinite parallel plates, d being the separation. For *small* separations the repulsion is certainly small compared with the VERWEY-OVERBEEK term.

But, since the latter repulsion falls away roughly as $exp(-\kappa d)$ for sufficiently large distances, the repulsion derived from F_t will then be the important term. LEVINE suggests that these repulsions might account for the very long range forces which seem to be required to explain tactoid formation.

In the sixth paper[60], the STERN theory of the layer of adsorbed ions is incorporated in the formalism set up in previous papers. This requires elaborate analysis; it is shown that the final effect is to add further terms to the usual VERWEY and OVERBEEK free energy. One of these corresponds to repulsion, and the other may be attractive or repulsive. Detailed calculations for *small* surface potentials show that repulsion predominates. These results are of interest and serve to demonstrate how complicated and difficult detailed calculations may be. It seems likely, however, that in practice the attractive terms arising from the electrical interaction cannot be of great importance, since otherwise the SCHÜLZE-HARDY rules would not be followed.

In the next paper, by LEVINE and SUDDABY[61], simplified forms for the free energy for two infinite parallel plates in an infinite symmetrical electrolyte, for the following special cases are given:

(*i*) Large separations (that is large compared with the double layer thickness);

(*ii*) Small separations;

(*iii*) Moderate potentials, and no restriction on the separation.

A further note[62] contains an extension of case (*i*).

This brings our account to the present day. It is of interest to look back and see how much of the original programme suggested in our list of possible improvements of our simple model of two point particles has been achieved. With regard to the interactions of finite particles, we have seen that there has been much work done on models of flat plates and spheres. Only one paper has dealt with other shapes, that of DUBE[63] on parallel cylinders but here the conclusions are spurious, again owing to the incorrect method of calculating the force due to LEVINE. Little has been done on the extension of the theory to a distribution unless LANGMUIR'S or ONSAGER'S work on coacervation, which will be reviewed presently, is included. Finally, no calculations have yet been made on the effect of dielectric saturation, or of image forces due to the discrete nature of the ions, or of their size. From the general success of the VERWEY-OVERBEEK theory, however, it seems unlikely that major alterations in the theory in its present state will eventually be required, although there is evidently plenty of scope for improvement in details.

With this we shall conclude our account of recent work on colloidal stability. The story is a good example of the scientific method in action with many false turnings and incomplete solutions before the final achievement. We now come to a group of less important effects which have not

been studied in such detail, but which have largely been elucidated, thanks to the work done in the field we have just surveyed.

4. *Mutual coagulation and stabilisation*

The complete precipitation of two stable sols can sometimes be brought about simply by thoroughly mixing the two. At one time it was thought that the precipitate was a chemical compound but it seems likely that in most cases it is simply a physical mixture of the solid components of the two sols from which it was formed. This effect is known as mutual coagulation. No detailed theory of it has been given. But the explanation is easy in the case where the particles of the two sols, say α and β, are oppositely charged. For although α—α and β—β pairs will repel each other and so be mutually stable, α—β pairs will exhibit attractive forces between the two components and so coagulate; the ionic double-layer merely modifies the mutual force.

More difficult to explain are the cases where the particles of the two sols have charges of the same sign. An example of this behaviour is afforded by arsenic trisulphide and sulphur sols. It may happen that chemical changes occur. But a purely physical explanation also seems possible. Even if the energy curves for α—α and β—β pairs are like curve I of Figure 3, for α—β pairs they might be of type II and so coagulation can take place.

An effect which may be regarded as the reverse of mutual coagulation is the ' stabilisation ' of a sol. Sometimes the addition of small quantities of another sol to an ordinary stable sol may make its subsequent coagulation far more difficult. A purely physical explanation of this effect is difficult to conjecture, and it seems likely that the added sol must produce surface changes in the main sol.

5. *The stability of emulsions*

Emulsions differ from sols and solid suspensions in that the dispersed phase is liquid not solid. The most important emulsions are oil-in-water or water-in-oil, the first word of the pair indicating the dispersed phase; the abbreviations *o/w* and *w/o* are commonly used. The size of the liquid globules in suspensions corresponds roughly to that of the particles in solid suspensions rather than that of particles in sols.

More specialized conditions are required for the formation of emulsions than for ordinary sols. The phase to be dispersed must usually be subjected to violent mechanical agitation; for example irradiation with ultrasonic waves. In general emulsions are not stable once the mechanical agent which has produced them is removed. Either suitable organic highly adsorbable ions, or finely divided solids, for example, graphite must be present. These are known as *emulsifying agents* or *emulsifiers*. Exceptions are emulsions of mercury which behave as ordinary sols.

Clearly, many of the qualitative results which have been derived for solid sols can be carried over to emulsions. No detailed calculations have been made; these would obviously be rather difficult since it would be necessary to take into account changes of free energy inside the droplets and possibly changes of shape. VERWEY[64] has pointed out however, that the theory of stability of solid sols explains the action of emulsifiers. Owing to the fact that the dielectric constants of most liquids which are immiscible in water are much less than that of water, it can be shown that, unless the liquid-liquid interface is highly charged, the potential of the drops is very small; consequently they would coalesce. Emulsifiers of the organic type provide charge for the interface. The action of solid emulsifiers is to coat the interface with a fine layer of solid particles so that the drop behaves, in its mechanical reactions with neighbouring drops, just like a solid (VAN DER MINNE[65]).

6. *The stability of foams*

In these the highly dispersed phase is a gas. Pure liquids do not form foams but require foaming agents. The action of these is similar to that of emulsifiers although the cases are not quite parallel since there cannot be a double-layer in the interior of a gas bubble.

7. *Schiller layers*

If suitably prepared sols of ferric oxide and a number of other metallic oxides are allowed to settle it is sometimes found that they show striking interference effects when illuminated from above. The effect is due to the sol, which is in the form of thin discs, forming parallel, horizontal layers in the liquid. The interference effects arise between light reflected from the different levels. The separation of the layers is of the order of a few thousand Ångstrom units.

BERGMANN, LÖW-BEER and ZOCHER[66] have given a satisfactory theory. The idea is that the layer formation is due to a balance between gravity which would tend to compress the layer system, and electrical repulsion due to double-layer interaction. The separation of the planes is so great that the VAN DER WAALS' attraction between them is not important.

We shall not give details of the calculations since the theory has already been covered. The formula of Eq. (3·15) in the limit of *small* interaction was used to give the electrostatic repulsion. The calculations gave reasonable values for the separations of the layers.

8. *Thixotropy and dilatancy*

Thixotropy is a property frequently exhibited by lyphobic gels. Under weak shearing stress the system behaves as a solid. For stronger stresses however, the elasticity vanishes and the material behaves as a fluid. On removal of the stress the viscosity increases with the time and eventually

the whole mass solidifies and returns to its gel state. Typical examples are bentonite gels, quicksand and thixotropic gels of ferric oxide.

No detailed theory of thixotropy on the lines of that developed for stability problems has been worked out but two main qualitative theories have been proposed. In the first it is suggested that the particles link up into a lattice-like open structure rather than coagulating as a whole in the ordinary fashion. For small stresses the framework will give rigidity to the whole mass; for large stresses the linkages will be broken and so ordinary fluid flow will result. When the stress is removed the lattice will gradually reform and so the rigidity will increase. Support for this view is afforded by the observation that thixotropy only seems to be found when the particles of the gel are dimensionally anisotropic. For example bentonite and ferric oxide gels are composed of thin plate-like particles. Clearly for the formation of a lattice this anisotropy is essential; a lattice formed from *spherical* particles would of necessity have a very high concentration of solid material whereas it is well-known that thixotropic gels may contain surprisingly small amounts of solid material by volume.

A somewhat different explanation is due to HAMAKER[67]. The regular structure is regarded as due to the secondary shallow minimum in the potential curves, Figure 3, curve I. According to VERWEY and OVERBEEK'S calculations however, the minima are very shallow and it is possible to argue that they are not sufficiently pronounced to give a stable lattice. It may happen however that for elongated particles in suitable mutual orientations, the minima are much more pronounced. A definite proof of the connection of thixotropy with the secondary minimum might be furnished by the observation of its occurrence on sols of roughly spherical shape since in this case the first theory would have to be ruled out.

Dilatancy, an effect which is almost the exact reverse of thixotropy is shown by many stable concentrated suspensions. Under weak stresses the system flows like a liquid, but for strong stresses high viscosity and even rigidity are exhibited. Dilatancy is very easily explained. For small stresses the electrical repulsion between the particles enables them to slip by each other easily. But as soon as the stress becomes sufficiently great they will be forced together and the system will behave like an ordinary solid in its elastic behaviour. Support for this view is provided by some observations by VERWEY and DE BOER[68]. They obtained strongly dilatant liquids simply by mixing fine powders and lubricating oils.

9. *Theory of the rate of coagulation of sols*

Suppose we have a sol in which the solid particles attract each other. Eventually the sol will coagulate, but the process will take a finite time and will not be instantaneous. This follows because each particle will, in general, spend a certain time before it meets that neighbour with which

it will eventually combine. Evidently the rate of coagulation will increase with the number of solid particles per unit volume and also the temperature, since raising the temperature will increase the Brownian motion. These ideas were put into mathematical form by SMOLUCHOWSKI[69,70]. He defined the coagulation rate G as the number of collisions experienced by a single particle in unit time and showed that, for the case in which there is no attraction except when the particles are in contact when it becomes infinite, the coagulation rate for a suspension of spheres of radius a, in a liquid of viscosity η, is

$$G = 8\,\pi\,a\,D\,N_0\,, \qquad \ldots\ldots (9{\cdot}1)$$

where D is the diffusion coefficient and N_0 the number of particles in unit volume. Using the EINSTEIN relation

$$D = \frac{kT}{6\pi\eta a}\,, \qquad \ldots\ldots (9{\cdot}2)$$

we find

$$G = \frac{4\,kT\,N_0}{3\eta}\,. \qquad \ldots\ldots (9{\cdot}3)$$

FUCHS[71] extended this to the case in which there is a more general interaction between the particles. If $V(r)$ denotes the mutual potential energy when the centres are r apart, then G should be divided by a factor W, where

$$W = 2\,a\int_{2a}^{\infty} \exp\left(V(r)/kT\right)\frac{dr}{r^2}\,. \qquad \ldots\ldots (9{\cdot}4)$$

This relation is of course only approximate. It implies that the relative motion of the coagulating particles is so slow that statistical equilibrium is always effectively maintained in the double-layer system round the particles. In this way the problem becomes one of pseudo-equilibrium and consequently can be treated with comparative ease. A rigorous analysis would be very difficult since it would be necessary to consider in detail the motion of the ions and of the electrolyte as the particles coalesced, and work out the mutual force at each stage of the motion.

Now let us return to FUCHS' theory. If V were evaluated, Eq. (9·4) could be used to calculate G and so, by comparison with experiment a check obtained on the nature of the V-r relation. The theory was first used however to throw light on the problem of stability. Evidently the condition for a very slow rate of coagulation, or in other words a relatively stable sol, is that W is very large.

DERJAGUIN[72] used for $V(r)$ the expression in Eq. (3·14) and concluded that for a very slow rate of coagulation the rate is mainly determined by the quantity

$$n = \epsilon a \psi_0^2 / kT \qquad \ldots . (9{\cdot}5)$$

which must be greater than 20 for stability.

VERWEY and OVERBEEK[73] pointed out that DERJAGUIN'S method was rather crude since proper account was not taken of VAN DER WAALS' forces between the particles, and in any case the result only applied for small surface potentials. Using their own values for the interaction energy which they had calculated in their work on the stability of sols containing spherical particles, they found W numerically from Eq. (9·4). They demonstrated that the main factor determining W was the height of the potential maximum in the plot of V as a function of r. For particles of diameter greater than about 10^{-5} cm, the integral was always so large that the height of the potential maximum was immaterial. This result confirmed in detail that their criterion for stability given in their discussion on the interaction of a pair of particles was satisfactory, provided that the dimensions were greater than 10^{-6} to 10^{-5} cms. For particles of sizes less than this, the smaller the particle the higher the maximum in the potential curve required for stability. Indeed, for particles less than 10^{-7} cm in radius, the surface potential necessary for stability is so high that it is doubtful whether it could ever be attained. This result clearly provides a very neat explanation of the difficulty of preparing extremely fine sols; for even when the desired state of subdivision is attained the sol will not in general be stable.

Another question discussed by VERWEY and OVERBEEK[73] by means of Eq. (9·4), was that of the deviations from the SCHÜLZE-HARDY rule.

For fairly rapid coagulation it is, of course, quite feasible to measure W experimentally. This was done by KRUYT and TROELSTRA[74] who measured the rates of coagulation of silver iodide sols under the influence of barium nitrate as electrolyte. Good agreement with some calculations made by VERWEY and OVERBEEK was obtained.

10. *Electrodeposition of suspensions*

So far we have considered two methods whereby the solid material in a sol or suspension can be separated in bulk from the suspending medium, namely depeptisation or flocculation by electrolytes. Another method of considerable industrial importance is electrodeposition. The method is used to coat objects with appropriate materials. An electric field is applied to a suspension of the particles to be deposited, the electrode to be coated being oppositely charged to the particles. It is found that coatings which adhere firmly are only produced with *stable* suspensions and rather high

field-strengths. Now if the suspension is stable it follows that the particles must repel one another. In this case it is obvious that we have a somewhat more complicated process than simply the transfer of material by the action of the electric field. For a particle arriving at the deposited layer might be expected to be repelled by it and so not deposited. Also one might expect an *unstable* suspension to form a better deposit since in this case the particles must attract one another.

VERWEY and HAMAKER[75] and also VERWEY and OVERBEEK[76] have shown how the main experimental facts can be explained from their theory of the interaction of sol particles. If suspensions giving satisfactory electrodeposition are allowed to stand for some time, the bottom of the vessel containing them becomes coated with a stable deposit just like the deposit formed by electrodeposition. This suggests that electrodeposition is not an effect specifically connected with the electric field; the field merely provides a force which drives the particles into close proximity. If the force is sufficiently great to overcome the repulsion between a particle already deposited and one moving up to it, the two will coalesce. The fields required to produce deposition on this theory turn out to be rather high, greater in fact than those used experimentally if it is assumed that the electrical resistance of the sol is uniform. It is accordingly suggested that, first a concentrated layer-like region of particles is built up near the surface. Since the electrical resistance of this layer will be greater than that the rest of the suspension there will tend to be a local increase in the field. This will continue until the local field is sufficiently intense to start deposition. On the other hand, in an unstable suspension the particles will tend to collect together to some extent before reaching the electrode, giving large loose aggregates from which a suitable firm coating cannot be formed.

All the applications of double-layer theory which we have examined so far have been closely connected with stability and the analysis of Section 3. We shall now consider three topics which require a viewpoint more concerned with the assembly as a whole. In one of these, the theory of micelles, the double-layer does not play an important part, but we have included a short account, since the calculations are very simple and may prove of interest to biophysicists.

11. *Soap micelles*

It has been known for a long time that soap solutions exhibit abnormally low osmotic pressures, often much less than if they contained simple undissociated molecules. But they also show high electrical conductivity. Since they are composed of salts of organic acids, this fact suggests ionic dissociation as in ordinary electrolytes. As MCBAIN pointed out in 1913 these properties could be explained if it were postulated that the large

organic ions aggregated; to the compound ions the name 'micelle' was given. This now seems to have become the accepted view and there are numerous theories and observations on size and shape.

DEBYE[77] has recently put forward an interesting method for determining the micelle size from measurements on light scattering by soap solutions. In addition a simple theory of micelle formation is presented. The micelles are viewed as bundles of ions rather like sheaves, with their carbon chains all parallel and the polar groups all at one end of the bundle in a plane. Suppose now two ions are brought together so that they lie parallel with their polar groups adjacent. In bringing them together energy is gained from the VAN DER WAALS' attraction, but lost owing to the mutual repulsion of the polar groups. For the whole micelle, the total energy due to the attraction will be roughly proportional to the total number of ions N in the micelle,

$$W_m = - Nw_m , \qquad \ldots . (11{\cdot}1)$$

where w_m is a constant. Next let us consider the electrical energy. For a circular disc of radius a, with charge density σ, the potential at the rim is of order σa. Hence the work done in increasing the radius by da is of order $\sigma^2 a^2 da$; so the total electrical energy varies as a^3. But the number of ions in the micelle must vary as a^2. Thus if W_e is the electrical energy

$$W_e = N^{\frac{3}{2}} w_e , \qquad \ldots . (11{\cdot}2)$$

where w_e is a constant.

The equilibrium size N_0 and total energy W_0 are evidently given by the conditions

$$dW/dN = 0 \quad , \quad W = W_e + W_m . \qquad \ldots . (11{\cdot}3)$$

Using Eqs. (11·1) and (11·2) we find

$$N_0 = (2w_m/3w_e)^2 \quad , \quad W_0 = - N_0 w_m/3 . \qquad \ldots . (11{\cdot}4)$$

N_o can be found experimentally and W_o derived by means of the law of mass action; the values obtained for w_e and w_m using Eqs. (11·4) agree reasonably well with other data.

Another fact which is neatly explained is the increase of micelle size accompanying addition of electrolyte to the solution. For as we have seen in the discussion of stability of sols, the effect of the double layer will be to reduce the electrical energy required to form the micelle. Hence w_e will be reduced and consequently, from Eq. (11·4), N_o will be increased.

12. *Solutions containing Zwitterions*

Solutions containing aliphatic amino acids with large molecules exhibit many of the usual properties of strong electrolytes. For example, their

osmotic pressure is quite normal. Rather surprisingly, however, they show abnormally low electrical conductivity. If the molecules were dissociated in the ordinary way one would expect the ions to behave as strong electrolytes, both in their activity and conduction.

KIRKWOOD[78] put forward the idea that the ions were hybrid. Although the resultant charge might be very low so that the conductivity was negligible, the intense local fields near the ionised groups on the surface would cause each to be surrounded by its double layer, and consequently, in respects other than conduction, the molecule would behave as an ordinary ion.

The following model was postulated for the ' Zwitterions ' as they were called. The molecule was taken to be a sphere of dielectric constant D_i of radius b containing M point charges at positions specified by the end points of vectors $\boldsymbol{r}_K$ drawn from the centre. To find the activity of a solution containing ions of this type it is necessary to calculate the free energy, and for this the appropriate potential solutions in and around the molecule must be worked out.

The potential V in the interior of the molecule may be written in the form

$$V(\boldsymbol{r}) = \sum_{k=1}^{M} \frac{e_K}{D_i \mid \boldsymbol{r} - \boldsymbol{r}_K \mid} + \psi \qquad \ldots . (12{\cdot}1)$$

where e_K is the charge at the point $\boldsymbol{r}_K$ and ψ is the contribution to the potential due to the solution. Outside the molecule two regions may be distinguished, a spherical shell between radii b and a, where a is the closest distance of approach for ions outside the molecule, and the region extending beyond the sphere of radius a. In the first region ($b < r < a$) the potential will be a solution of LAPLACE'S equation; in the second region it must satisfy the DEBYE-HÜCKEL equation, Eq. (2·8). The general solutions for the potentials in the two regions are easily written down. From the boundary conditions, which must hold at the two spherical surfaces which separate the three regions, it is possible to give the potential solution completely in terms of the set of charges e_K and their positions $\boldsymbol{r}_K$ in the molecule, although the analysis is cumbersome in consequence of the lack of spherical symmetry. Formal expressions for the field energy, free energy, chemical potential and activity are easily obtained from the potential. In this way, KIRKWOOD was able to show that the solution containing Zwitterions would have many of the usual properties of strong electrolytes, even if the net charge on the ions were zero.

Later KIRKWOOD and WESTHEIMER[79] used the theory to account for the influence of substituents on dissociation constants of organic acids. The substituents were regarded as cavities of low dielectric constant in the dielectric sphere of radius b. The theory was also extended to molecules of

ellipsoidal shape. Obviously a *sphere* of dielectric might not be a good model for many acids.

13. *Coacervation*

This phenomenon bears a strong resemblance to the coagulation of sols. For appropriate ranges of concentration of the sol and electrolyte, instead of the sol going completely into a solid phase it forms regions of high concentration called tactoids. When these coalesce a separate liquid phase is formed. The effect only seems to be produced in sols for which the extension of the particles in one or two dimensions is great; for example, with thin discs or rods. In the phase of higher concentration, or coacervate, the particles follow a loose regular structure which gives the phase anisotropic optical properties. The best known example is the tobacco mosaic virus sol. A 2 per cent solution of this virus in ·005 N, NaCl solution forms separate phases. In the anisotropic phase the protein rod-shaped molecules are roughly arranged in a two dimensional hexagonal lattice.

LANGMUIR[40] first put forward a theory of the formation of coacervates. He rejected the accepted view that VAN DER WAALS' attractive forces were of importance in colloidal sols. The starting point of his analysis is the formula given by DEBYE and HÜCKEL for the osmotic pressure of strong electrolytes.

$$p = kT \sum_{i=1}^{s} n_i - \tfrac{1}{3}\left(\frac{\pi e^6}{\epsilon^3 kT}\right)^{\frac{1}{2}} \left(\sum_{i=1}^{s} n_i z_i^2\right)^{\frac{3}{2}} . \quad \ldots . (13{\cdot}1)$$

T is the temperature, n_i the ionic concentration of the ions, z_i their valency. The structure of the ions is ignored; they are merely regarded as point charges. The summations are over all the ionic species present. LANGMUIR now assumes that this expression can be carried over without modification to sols, and the sol particles themselves treated on the same footing as ordinary ions. The simplest possible case would be that in which we had the sol with charge z_1 and concentration n_1, and a single ion of the ordinary type present, of valency z_2 and concentration n_2. The condition for electro-neutrality gives

$$n_1 z_1 + n_2 z_2 = 0 . \quad \ldots . (13{\cdot}2)$$

Eliminating n_2 between Eq. (13·1), in which s is set equal to 2, and Eq. (13·2), we find

$$p = \left(1 - \frac{z_1}{z_2}\right) n_1 kT - \frac{1}{3}(\pi e^6/\epsilon^3 kT)^{\frac{1}{2}} z_1^{\frac{3}{2}} (z_1 - z_2)^{\frac{3}{2}} n_1^{\frac{3}{2}} , \quad \ldots . (13{\cdot}3)$$

or, for a given sol and temperature, the dependence of p on the sol concentration is of the form

$$p = a_1 n_1 - a_2 n_1^{\frac{3}{2}} , \quad \ldots . (13{\cdot}4)$$

where a_1 and a_2 are independent of n_1.

Suppose now that p is plotted as a function of n_1. The osmotic pressure rises from zero to a maximum and then falls to zero again (curve I, Figure 4). LANGMUIR next suggested that for concentrations greater than the maximum of curve I the charge on the sol particles falls, since their surface potential must be regarded as remaining unchanged. Since from Eqs. (13·1) and (13·4) it follows that a_2 is very nearly proportional to z_1^3, the negative contribution in Eq. (13·4) begins to fall rapidly as n_1 increases. Under favourable circumstances, therefore, curve I would be changed to curve II for high concentrations. Suppose now we arranged for a gradual increase of concentration of sol from zero; the osmotic pressure would follow the curve I until the point M were reached, M having the same ordinate as the *minimum* on curve II. From this point two concentrations can give the same osmotic pressure, one on curve I, the other on curve II. This means physically that two phases can co-exist, in one the concentration being much greater than in the other; in other words, coacervation is explained.

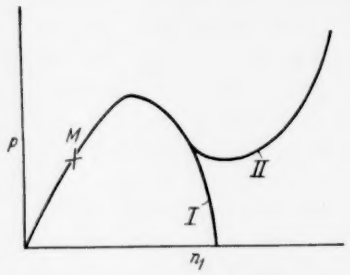

Figure 4. Osmotic pressure as a function of sol concentration according to Langmuir's theory.

Although the above theory provides a neat, qualitative explanation of coacervation, serious objections to it have been raised[80]. The disparity in size between ordinary ions and sol particles is so great that it is extremely doubtful if it is legitimate to apply Eq. (13·1) to the system as a whole, including the sol with the ordinary ions. The theory would, however, be on much firmer ground if we were considering sol particles of extremely small size, and so might be useful when applied to some systems.

Very recently another analysis has been given in a long memoir by ONSAGER[81]. His contention is that the remarkable properties of sols exhibiting coacervation are due primarily to their characteristic *shape*; sols of the same material made up of *spherical* particles with the same volume of solid in the same volume of liquid would not show any remarkable coacervation properties. The ionic double layer of the sol is introduced in a rather rough way; its resultant effect is equivalent to a modification of the sol dimensions.

Suppose the sol contains N particles, the whole configuration being specified by sets of coordinates (q_1), (q_2) . . . . . (q_N). For point particles three ordinary space coordinates would suffice for each (q_r), but for asymmetrical particles further coordinates will be required to specify orientation. If w_N ((q_1), (q_2), ... (q_N)) is the potential of the average

forces between the particles, then, by a general theorem of statistical mechanics[82], the osmotic pressure of the particles is given by the relation

$$p = kT \frac{\partial (\log B)}{\partial V}, \qquad \ldots . (15{\cdot}5)$$

where T is the temperature, k BOLTZMANN'S constant, V is the volume, and B is defined by

$$B(N, V, T) = \frac{1}{\underline{N}} \int \exp(-w_N/kT) \prod_{r=1}^{N} (d\ q_r) . \qquad \ldots . (13{\cdot}6)$$

The integral in the last equation is taken over all the coordinates, $(q_1) \ldots . (q_N)$ corresponding to the spatial positions of the particles which must be confined to the volume V. It follows that provided B can be calculated, p can be determined.

ONSAGER then proceeds to find the function B by the MAYER and MAYER method[83], neglecting attractive forces of the VAN DER WAALS' type and electrical repulsion due to double-layer interaction, leaving only contact repulsive forces. For these we can write

$$w_N((q_1), (q_2), \ldots . (q_N)) = \sum_{i<j} w_{ij} , \qquad \ldots . (13{\cdot}7)$$

where w_{ij} is short for $w_2((q_i), (q_j))$.

Now let

$$\Phi_{ij} = \Phi((q_i), (q_j)) = e^{-w_{ij}/kT} - 1 . \qquad \ldots . (13{\cdot}8)$$

Then we can expand $\exp(-w_N/k\ T)$ in series, involving the Φ_{ij}.

Suppose for the moment that the asymmetry of the particles is ignored and we take the forces between them as central ones. Then B is given in terms of the Φ's by the relation

$$\log B = N(1 + \log(V/N)) + N^2 \beta_1/2V + N^3 \beta_2/3\ V^2 \qquad \ldots . (13{\cdot}9)$$

The β_n are the so-called 'cluster integrals' given by

$$\beta_1 = \frac{1}{V} \iint \Phi_{12}\, d\tau_1\, d\tau_2 ,$$

$$\beta_2 = \frac{1}{2V} \iiint \Phi_{12}\, \Phi_{23}\, \Phi_{31}\, d\tau_1\, d\tau_2\, d\tau_3 ,$$

$$\beta_3 = \quad - \quad - \quad - \qquad \ldots . (13{\cdot}10)$$

The coordinates τ_1, τ_2 and τ_3 are the ordinary space coordinates of the centres of the particles. The integrations are taken throughout the volume V. If the N particles are composed of different species, each denoted by

the running suffix s, formula (13·9) generalizes to

$$\log B = \Sigma N_s (1 + \log (V/N_s)) + \frac{1}{2V} \sum_{s,s'} \beta (s, s') N_s N_s' + \frac{1}{3V^2} \sum_{s,s',s''} \beta_2 (s, s', s'') N_s, N_{s'} N_{s''} + \quad \ldots . (13{\cdot}11)$$

where N_s denotes the number in species s. β_1 (s, s') denotes the integral of Eq. (13·10), in which one particle is of species s, the other of species s'. β_2 (s, s', s'') is defined in a similar fashion.

Now let us return to the problem of asymmetric particles. The obvious method of proceeding would be to take over formula (13·9) and generalize the cluster integrals. ONSAGER used a different method, however. He introduced the artifice that particles with different orientations are to be regarded as of a different kind and the second formula (13·11) used. Suppose the totality of all directions in space is divided into elements of solid angle $\triangle\Omega_1$ $\triangle\Omega_2$ . . . . . $\triangle\Omega_s$, surrounding directions $\boldsymbol{a}_1$, $\boldsymbol{a}_2$ . . . . . $\boldsymbol{a}_s$. If $\triangle N_\nu$ denotes the number of particles with suitably defined axes in direction $\boldsymbol{a}_\nu$, then appropriate distribution functions can be defined by the equations

$$\triangle N\nu = N f (\boldsymbol{a}_\nu) \triangle \Omega_\nu . \quad \ldots . (13{\cdot}12)$$

Using Eq. (13·11) for $\log B$, we find

$$\log B = \sum_\nu \triangle N_\nu (1 + \log (V \triangle \Omega_\nu / 4\pi \triangle N_\nu)) + \frac{1}{2V} \sum_{\nu,\nu'} \beta_1 (\boldsymbol{a}_\nu, \boldsymbol{a}_{\nu'}) \triangle N_\nu \triangle N_{\nu'} + \quad \ldots . (13{\cdot}13)$$

Replacing summations by integrations we finally have

$$\log B = N \left[1 + \log (V/N) - \int f (\boldsymbol{a}) \log 4\pi f (\boldsymbol{a}) \, d\Omega + \frac{N}{2V} \iint \beta_1 (\boldsymbol{a}, \boldsymbol{a}') f (\boldsymbol{a}) f (\boldsymbol{a}') \, d\Omega \, d\Omega' + 0 \left(\frac{N}{V}\right)^2 \right], \quad (13{\cdot}14)$$

where

$$\int f (\boldsymbol{a}) \, d\Omega = 1 , \quad \ldots . (13{\cdot}15)$$

All the integrations are taken over all possible spatial directions.

To make further progress it is necessary to evaluate the β_n functions. This is a geometrical problem; in an appendix of the paper ONSAGER evaluates β_1 for the case of two cylinders of length l_1 and l_2 and diameters d_1 and d_2 with their axes inclined at an angle γ. The calculation of β_2 and

the higher order cluster integrals is too complicated to be accomplished completely, but it is possible to give estimates of the averages of β_2 over the angles in terms of the average of β_1.

The next step is to see how β_1 is affected when account is taken of the double layer repulsion. ONSAGER uses an approximation due to VERWEY and OVERBEEK and shows that the effect is equivalent to an increase in the diameters of the cylinders by δ where δ is the solution of the equation

$$w_2(\delta) = kT\, e^{-c} . \qquad \ldots . (13{\cdot}16)$$

$w_2(\delta)$ denotes the energy when the separation of the axes is δ and C is EULER'S constant. The final expression for the value of β_1 (averaged over the angle γ), if d_1 and d_2 are equal, is

$$\bar{\beta}_1 = -\pi\, l_1\, l_2\, (d + \bar{\delta})/2 , \qquad \ldots . (13{\cdot}17)$$

where

$$\bar{\delta} = \kappa^{-1}\left[c + \log a + \log 2 - \frac{1}{2}\right], \qquad \ldots . (13{\cdot}18)$$

and a depends only on the electrolyte.

We are now in a position to discuss the application of our principal formula (13·14) to sols. First let us examine isotropic solutions. For these any direction for the axes of the particles is equally probable. This means that in Eq. (13·14) we can replace the integrals by the averages, since

$$f(\alpha) = 1/4\pi . \qquad \ldots . (13{\cdot}19)$$

The final formula for B in Eq. (13·14) is

$$\log B = N\,[1 + \log (V/N) - b\,(N/V)], \qquad \ldots . (13{\cdot}20)$$

where

$$b = \pi\, l^2\, (d + \bar{\delta})/4 , \qquad \ldots . (13{\cdot}21)$$

for long rods of length l, diameter d, and for thin discs of diameter d

$$b = \pi^2\, d^3/16 . \qquad \ldots . (13{\cdot}22)$$

It is interesting to compare Eqs. (13·21) and (13·22) with the corresponding formula for spheres in which b is the well-known co-volume and is simply four times the volume. For the two cases above it is clear that the covolume can be of quite a different order of magnitude from the volume. For example, the volume for the case of Eq. (13·21) would be of order $l\, d^2$, whereas b is of order of $l^2 d$. The ratio of the second order to the first is l/d. Hence a much lower concentration of sol is required to produce a given change in the osmotic pressure than if the particles were spherical. Another interesting feature is the presence of $\bar{\delta}$ in Eq.

(13·21). Under suitable conditions $\bar{\delta}$ may be large compared with d, and so the covolume may be strongly dependent on the concentration of electrolyte. This follows, since from Eq. (13·18) it is seen that $\bar{\delta}$ is roughly proportional to the double-layer thickness.

From Eq. (13·20) the derivation of the osmotic pressure and the chemical potential is straightforward. The formulae are also easily extended to suitable *polydisperse* solutions; that is, solutions with particles of different dimensions. One result of physical interest is that for long thin rods of the same diameter but of varying lengths, the correction for the covolume depends only on the total length of the rods in unit volume of solution, not upon the way in which the lengths are distributed.

So far we have only discussed the *isotropic* solution of the fundamental equation, Eq. (13·14). The more interesting case arises however when the distribution function $f(\boldsymbol{a})$ is *non-isotropic*, that is, when there is a tendency for the axes of the particles to align along a particular direction or set of directions. This corresponds closely with the observed structure of coacervates which often seems to be that of nematic liquid crystals. In ordinary crystals there is a regular distribution in space. But in nematic liquid crystals the distribution in *space* is random, but the distribution of the orientation is not.

The following problem now presents itself. If in fact $f(\boldsymbol{a})$ is non-isotropic how can we ascertain its form? Following the usual thermodynamical theory we must select that distribution function $f(\boldsymbol{a})$ which makes the HELMHOLTZ free energy F of the system a minimum under conditions of constant temperature. But the free energy is related to the function B of Eq. (13·6) by the equation

$$F = -kT \log B \; . \qquad \ldots . (13{\cdot}23)$$

It follows, therefore, that we must maximize B with respect to $f(\boldsymbol{a})$, subject to the normalizing condition

$$\int f(\boldsymbol{a})\, d\Omega = 1 \; . \qquad \ldots . (13{\cdot}24)$$

If we drop terms in Eq. (13·14) involving the troublesome integrals of β_2, β_3 *etc.* and so restrict our analysis to small concentrations, the solution of this variational problem is easily found by the familiar LAGRANGE method of undetermined multipliers. The solution is obtained in the form of an integral equation for $f(\boldsymbol{a})$

$$\log 4\pi f(\boldsymbol{a}) = \lambda - 1 + c \int \beta_1(\boldsymbol{a}, \boldsymbol{a}') f(\boldsymbol{a}')\, d\Omega' \quad \ldots . (13{\cdot}25)$$

where λ is a constant and c is the concentration, that is N/V. One can easily verify that this equation can be satisfied for an isotropic solution. For we have Eq. (13·19) for f($\mathbf{a}'$) and Eq. (13·25) is satisfied provided that

$$\lambda = 1 + 2\, b\, c \; . \qquad \ldots . (13{\cdot}26)$$

ONSAGER discusses non-isotropic solutions in some detail and shows that it is perfectly possible to construct them for sufficiently high concentrations. Further, suppose the concentration c is increased from zero to a sufficiently high value for the distribution to become markedly anisotropic. Then, will the solution change over wholly from the isotropic to anisotropic state, or will there be a region of concentration in which isotropic and anisotropic phases can coexist? Using very general arguments the second possibility, which of course is the one found in practice, seems to be favoured. If it is assumed that the two phases coexist then it is possible to form some estimate of the relative concentrations of the particles in the two phases. First, however, it is necessary to find $f(\boldsymbol{a})$ from Eq. (13·25). As it stands the equation is intractable and it is necessary to assume suitable trial functions with parameters and then adjust the values of these parameters for the best fit. ONSAGER takes the form

$$f(\boldsymbol{a}) = \frac{\alpha}{4\pi} \sinh \alpha \cosh \alpha \, (\boldsymbol{a}.\boldsymbol{a}_0) , \qquad \ldots . (13{\cdot}27)$$

where α is the parameter and $\boldsymbol{a}_0$ is a unit vector in a fixed direction. $(\boldsymbol{a}.\boldsymbol{a}_0)$ is simply the scalar product of the two vectors. If the rods are mainly orientated in directions in a fairly small solid angle about the direction $\boldsymbol{a}_0$, from Eq. (13·27) we see that α must be very large. This simplifies the calculations considerably.

We can now return to the question of the equilibrium between the isotropic and anisotropic phases. Clearly the conditions of equilibrium will be that both the osmotic pressures and chemical potentials for the two phases are identical; both of these quantities are easily calculated from the general formula for B. Hence if c_a and c_i denote the concentrations in the anisotropic and isotropic phases respectively, we have two equations from which, on eliminating α, the ratio c_a/c_i is found. The equations were solved numerically and gave the value 1·34. This is the order of magnitude of the experimentally observed ratio.

Further developments are due to ISAHARA[84,85], who examined the theory of suspensions of spheroidal and dumb-bell particles. Simple criteria for the concentrations at which coacervation would appear, were given. Since double-layer effects were not introduced however, we shall not go into the details of the calculations.

With this account of coacervation we shall conclude our discussion of topics which can be dealt with by the statistical mechanical techniques for systems in equilibrium. Now we must pass on to consider problems in which thermodynamic equilibrium is not established. First we shall examine the theory of streaming potentials and electro-osmosis; this is rather simpler than the theory of other electrokinetic effects.

14. *Streaming potentials and electro-osmosis*

The principal developments in this field concern extensions of the older theory to include surface conduction effects. If an electric field is applied to an electrolyte in a capillary or diaphragm, or closely packed powder, the electrolyte in general flows along the field direction. When the external pressure gradient along the capillary is zero, so that the liquid is at rest when no field is applied, then it is generally found that the rate of flow is proportional to the field strength. The flow obtaining under these conditions is known as '*electro-osmosis*.' On the other hand, if the electrolyte is forced through the capillary by an external pressure difference, it is found in general that the emerging liquid is charged. The charge transferred from the source of the electrolyte to the receiving sink can be modified by applying a field along the axis of flow. The potential gradient along the capillary required to prevent transfer of charge is known as the '*streaming potential*.' Both effects arise from the double layer at the walls of the capillary. Owing to their simplicity both have been used very extensively to measure zeta potentials.

Let us first consider the streaming potential. For the case of cylindrical tubes, the walls of which have a uniform surface charge density, the relation between the streaming potential and applied pressure gradient can be found very simply as follows. If r denotes the distance of a point on the capillary from the axis, and a is the radius, the velocity distribution parallel to the axis is, from the well-known POISSEUILLE theory

$$v\,(r) = \frac{P}{4\eta l}\,(a^2 - r^2)\,, \qquad \ldots\ldots (14{\cdot}1)$$

where P/l is the pressure gradient and η the coefficient of viscosity. The charge carried along the tube per unit time is

$$I_1 = \pi \int_0^a v\,(r)\,\rho\,(r)\,r^2\,dr\,, \qquad \ldots\ldots (14{\cdot}2)$$

where $\rho\,(r)$ is the charge density on the double layer. If we now apply the restriction that the double layer is thin compared with the radius of the tube, so that ρ is only significant in a thin layer near the wall, I_1 becomes

$$I_1 = 2\pi a \int_0^\infty \rho\,(x)\,v\,(x)\,dx\,, \qquad \ldots\ldots (14{\cdot}3)$$

where

$$x = a - r\,.$$

For values of x small compared with a, Eq. (14·1) gives

$$v\,(x) = Pax/2\,\eta\,l\,. \qquad \ldots\ldots (14{\cdot}4)$$

Substituting in Eq. (14·3), and using the relation

$$\frac{d^2\,\psi}{dx^2} = -\,\frac{4\pi\rho\,(x)}{\epsilon}\,, \qquad \ldots\ldots (14{\cdot}5)$$

where ψ is the potential in the double layer and ϵ the dielectric constant, we find on integration

$$I_1 = -\,\frac{\epsilon\,\zeta\,a^2\,\eta\,P}{4\,\eta\,l}\,, \qquad \ldots\ldots (14{\cdot}6)$$

where ζ is the zeta potential of the boundary.
If there is an applied field E/l along the axis, however, there will be an additional current I_2 owing to the ordinary conduction process,

$$I_2 = \frac{\pi\,a^2\,\sigma\,E}{l}\,, \qquad \ldots\ldots (14{\cdot}7)$$

where σ is the ordinary electrical conductivity of the electrolyte in the capillary. The condition

$$I_1 + I_2 = 0 \qquad \ldots\ldots (14{\cdot}8)$$

now gives for the ratio of the streaming potential to the pressure gradient

$$\frac{E}{P} = \frac{\epsilon\,\zeta}{4\pi\,\eta\,\sigma}\,. \qquad \ldots\ldots (14{\cdot}9)$$

RUTGERS[86] pointed out that Eq. (14·9) would not be correct if the compact double layer possessed a high conductivity. In fact if σ_s is the specific surface conductivity, instead of Eq. (14·7), we have

$$I_2 = E\,(\pi a^2\,\sigma + 2\pi\,a\,\sigma_s)/l\,, \qquad \ldots\ldots (14{\cdot}10)$$

and we find

$$\frac{E}{P} = \frac{\epsilon\,\zeta}{4\pi\,\eta\,\sigma\left(1 + \dfrac{2\,\sigma_s}{a\,\sigma}\right)}\,. \qquad \ldots\ldots (14{\cdot}11)$$

Evidently if a is sufficiently small, and σ_s sufficiently large compared with σa, the correction term may become of importance. Also the zeta potential calculated from Eq. (14·9) will show a variation with a which is evidently spurious. RUTGERS used Eq. (14·11) to analyse data on streaming potentials in glass capillaries and found surprisingly high values of the

surface conductance. More recent work has indicated much lower values, however, so that the effect may be of less importance than was thought at first[87].

The question of the application of RUTGER'S formula to streaming through capillaries not necessarily cylindrical or of uniform cross-section, for example, diaphragms or closely packed powders, has been examined by OVERBEEK and WIJGA[88]. It is easy to show that a close relation holds between electro-osmosis and the streaming potential. If V is the volume transported through the capillary in unit time by electro-osmosis (the external pressure gradient being zero) when the charge transported is I, then

$$\frac{E}{P} = \frac{V}{I} \quad , \qquad \ldots . (14{\cdot}12)$$

both for the case when surface conductance is present, and when it is absent. OVERBEEK and WIJGA in their paper point out that although Eqs. (14·11) and (14·12) have only strictly been derived for cylindrical capillaries, a number of authors have used them to interpret experiments with diaphragms, that is systems which may be regarded as collections of capillaries of varying lengths and diameters and with interconnections. The question arises as to whether Eqs. (14·11) and (14·12) are still valid for the more complicated systems of this type. The answer given by the above authors is that in general Eq. (14·12) may be valid, but Eq. (14·11) is incorrect unless surface conductance can be ignored.

It is first shown that Eqs. (14·11) and (14·12) are still followed for a system consisting of two cylindrical, parallel capillaries of different radius. For two capillaries of different diameters in series, however, Eq. (14·11) is no longer true. If transport of charge by surface conductance is preponderant it can be shown that the ratio of the zeta potential calculated from Eq. (14·11) to the true value is

$$\frac{\zeta^1}{\zeta} = \frac{\left(\frac{l_1}{r_1^3} + \frac{l_2}{r_2^3}\right)\left(\frac{l_1}{r_1^2} + \frac{l_2}{r_2^2}\right)}{\left(\frac{l_1}{r_1^4} + \frac{l_2}{r_2^4}\right)\left(\frac{l_1}{r_1} + \frac{l_2}{r_2}\right)} \quad , \qquad \ldots . (14{\cdot}13)$$

where l_1 and l_2 are the lengths of the capillaries, r_1 and r_2 their radii. This ratio is always *less* than unity, which probably accounts for the fact that zeta potentials calculated from Eq. (14·11) from data using diaphragms seem to be too low. Thus although a diaphragm is more complicated than either a system of capillaries of different radii in series or parallel, it is evident that Eq. (14·11) cannot be used for diaphragms or powdered material if there is appreciable surface conduction.

In the first paper OVERBEEK and WIJGA only proved Eq. (14·12) for two simple models, capillaries of different radii in series or parallel. A second paper due to OVERBEEK and MAZUR[89] is concerned with an arrangement more like a real diaphragm; that is, a system of capillaries with interconnections. They show by two methods that Eq. (14·12) is still valid for this arrangement. The first step in the first method is to obtain a general expression for the rate of electro-osmosis through a single capillary of uniform cross-section, but of arbitrary form. It is shown that if V is the rate of transport of fluid when a potential E is established between the ends, distant l apart, then

$$V = \frac{\epsilon \zeta E C}{4\pi\eta}, \qquad \ldots\ldots (14{\cdot}14)$$

where

$$C = \frac{1}{l}\int \left(1 - \frac{\psi}{\zeta}\right) d S. \qquad \ldots\ldots (14{\cdot}15)$$

The integral of Eq. (14·15) is taken over the cross-section S of the capillary. ψ is the potential in the electrolyte when the liquid is at rest. The quantity C is evidently a function of the capillary dimensions and of the double layer structure. Except for very fine capillaries whose dimensions are roughly of the same order as the double layer thickness, it reduces simply to the cross-section divided by the length. This follows since, provided the double layer thickness is small compared with the cross-sectional dimension, the integral of Eq. (14·15) will simply be unity except in a small area—the double-layer region—near the boundary. The next step is to show that for the same capillary, but with a pressure difference P applied to the ends instead of a potential difference, the streaming current is given by

$$I = \frac{\epsilon \zeta P C}{4\pi\ \eta}\ . \qquad \ldots\ldots (14{\cdot}16)$$

The fact that the constant multiplying $\epsilon\zeta/4\pi\eta$ in both Eqs. (14·15) and (14·16) involves the same function C, is an essential step in the subsequent analysis. Using Eqs. (14·15) and (14·16) OVERBEEK and MAZUR then show that for a network of capillaries, to the ends of which both a pressure P and potential difference E are applied, E, P, the streaming current I, and the liquid flow in unit time V, are related by the equations

$$\begin{aligned} E &= AI + BV, \\ P &= BI + DV, \end{aligned} \qquad \ldots\ldots (14{\cdot}17)$$

where A, B and D are coefficients determined only by the structure of

the diaphragm and the liquid-solid interfaces. They are related in a rather complicated way to the electrical and hydrodynamic conductivities of the individual capillaries, the zeta potentials and the various constants C, defined by Eq. (14·15). Eq. (14·12) follows easily, for we have

$$\left(\frac{E}{P}\right)_{I=O} = \frac{B}{D} \quad , \qquad \ldots . (14{\cdot}18)$$

and

$$\left(\frac{V}{I}\right)_{P=O} = \frac{B}{D} \, . \qquad \ldots . (14{\cdot}19)$$

Other relations can be derived from Eqs. (14·17); for example

$$\left(\frac{E}{V}\right)_{I=O} = \left(\frac{P}{I}\right)_{V=O} = B \quad , \qquad \ldots . (14{\cdot}20)$$

$$\left(\frac{I}{P}\right)_{E=O} = \left(\frac{V}{E}\right)_{P=O} = \frac{B}{B^2 - AD} \, . \qquad \ldots . (14{\cdot}21)$$

The second method employs the recent theory of the thermodynamics of the steady state of ONSAGER[90]; it requires no particular model for the diaphragm. If the currents I and V are linearly dependent on the applied potentials E and P, a fact which may be justified experimentally, we can write

$$\begin{aligned} I &= L_{11} E + L_{12} P \, , \\ V &= L_{21} E + L_{22} P \, , \end{aligned} \qquad \ldots . (14{\cdot}22)$$

where the constants L_{rs} depend only on the structure of the capillaries. Applying ONSAGER'S reciprocity theorem, we now find that

$$L_{12} = L_{21} \, . \qquad \ldots . (14{\cdot}23)$$

This enables Eq. (14·22) to be written in exactly the same form as Eq. (14·17) and so the same conclusions follow.

A further very recent application of ONSAGER'S method which is closely related to the analysis of OVERBEEK and MAZUR is due to STAVERMAN[91]. In this paper the inter-relation of three types of membrane processes, namely electro-osmosis, ordinary osmosis and the establishment of diffusion potentials is closely studied. A considerable number of new phenomenological relations are derived, and it is shown that the behaviour of a membrane can be completely specified by a number of suitably defined parameters without any reference to particular models of the structure of the membrane.

15. *Electrophoresis*

Electrophoresis is a general term for the motion of matter in a liquid, the motion being caused by an externally applied electric field. The most important case arises when the suspended matter is solid although the electrophoresis of droplets of liquids and gas bubbles is also of interest. Electro-osmosis and electrophoresis are very closely related. Both can be regarded as due to the relative motion of liquid and solid when an electric field with a component parallel to the boundary between the two, is applied to the system. If the solid is regarded as fixed then the field produces motion of the liquid and we have electro-osmosis. On the other hand, if the solid is free and the liquid in bulk regarded as stationary, we get electrophoresis. It was this close relationship between the two effects that enabled SMOLUCHOWSKI[92] to deduce a relation connecting the rate of electrophoresis and the external field from HELMHOLTZ's analysis of electro-osmosis.[2] His classical formula giving the rate of electrophoresis U of a solid particle in a liquid is simply

$$U = \frac{\epsilon \zeta E}{4\pi \eta} . \qquad \ldots . (15{\cdot}1)$$

ζ is the zeta potential of the surface, the meaning of this term being that given in Section 1, ϵ the dielectric constant of the electrolyte, E the applied field, and η the coefficient of viscosity of the liquid. The formula is remarkable in that it does not depend on either the size or shape of the particle, but it is important to notice that it only holds under rather restricted conditions;

1. The double layer thickness is much less than the radius of curvature at any point of the surface.

2. The particle is non-conducting.

3. Both ϵ and η are uniform throughout the double-layer

and 4. It is assumed that the distribution of changes in the ionic double layer is not affected by the applied field.

Unfortunately SMOLUCHOWSKI's formula is inadequate for application to many particles of interest for the reason that they are too small for condition 1. to apply. In extreme cases, of course, the particle size may be much *less* than the double layer thickness. In this case it is legitimate to ignore double-layer effects. The problem then reduces simply to one of ordinary hydrodynamics; we require the rate of steady motion of the body through the uncharged fluid when acted upon by a force E times the total surface change. This will now, of course, depend on the shape. The simplest case is that of a sphere for which the well-known STOKES solution holds; the only change in Eq. (15·1) is the replacement of 4 in the denominator by 6.

When the double-layer thickness is neither very small nor very large compared with the size, the only method is to examine the flow round the particle in detail. This makes the problem much more difficult than in SMOLUCHOWSKI's theory; in consequence little work has been done on shapes other than spheres.

(*a*) *Solid spherical particles*—The first important paper in the period under review is due to HENRY[93] who gave a formula for U without SMOLUCHOWSKI's restrictions 1 and 2, but with the additional condition that the zeta potential was sufficiently small to make $e\,\zeta/k\,T$ small compared with 1. This condition is not required in SMOLUCHOWSKI's analysis. HENRY found for U the formula

$$U = \frac{E\,\epsilon\,\zeta}{6\pi\,\eta}\,(1 + \lambda\,F(b))\,, \qquad \ldots\ldots (15{\cdot}2)$$

where

$$\lambda = \frac{\sigma_0 - \sigma}{2\sigma_0 + \sigma}\,, \qquad \ldots\ldots (15{\cdot}3)$$

σ being the conductivity of the solid material of the particles σ_0 of the electrolyte, and where

$$F(b) = \frac{1}{8}\,b^2 - \frac{5}{24}\,b^3 - \frac{1}{48}\,b^4 + \frac{1}{48}\,b^5 + \frac{1}{4}\,b^4\,e^b\,E_i(b)\left(1 - \frac{1}{12}\,b^2\right),$$

with

$$E_i(b) = \int_b^\infty \frac{e^{-x}\,dx}{x}\,. \qquad \ldots\ldots (15{\cdot}4)$$

b equals κa and so represents the ratio of the radius to the double layer thickness.

For non-conducting particles λ equals $\frac{1}{2}$. For b large, that is for a double-layer thickness *small* compared with the radius, $F(b)$ tends to 1, and so Eq. (15·2) gives Eq. (15·1). On the other hand, for b small, that is for a double layer thickness very *large* compared with the radius, $F(b)$ tends to zero and we again get a result already found. The function $F(b)$ increases monotonically from 0 for b small, to 1 for b large.

Now let us consider the derivation of formula (15·2). At first sight it might appear that the calculation of U would be almost trivial. For the force on the sphere one simply has the charge multiplied by the field; this must be equated to the viscous resistance given by STOKES' law. A calculation on these lines would be quite fallacious, however, for the following reason. The electrolyte near the surface, owing to the ionic

atmosphere, bears a charge opposite in sign to that on the surface. Hence, owing to the field it tends to flow in the opposite direction to the particle. The resistance experienced by the particle is *greater* than would follow from the STOKES' solution. In fact, if the counterflow in the double layer is ignored it is easily shown that, if ζ is kept fixed, the velocity U would tend to infinity as the double layer thickness tended to zero, instead of giving Eq. (15·1).

Now let us examine HENRY's derivation of Eq. (15·2) in some detail. For steady motion and axes fixed with respect to the centre of the sphere, for the electrolyte the equations of motion and of continuity respectively are written in the form

$$\eta \operatorname{curl}^2 \boldsymbol{v} + (\boldsymbol{v}.\operatorname{grad} \boldsymbol{v}) + \operatorname{grad} p + \rho \operatorname{grad} \psi = 0 , \quad \ldots . (15{\cdot}5)$$

$$\operatorname{div} \boldsymbol{v} = 0 \quad . \quad \ldots . (15{\cdot}6)$$

η is the coefficient of viscosity of the electrolyte, $\boldsymbol{v}$ the fluid velocity, p the pressure, ρ the charge density, and ψ the electrical potential. Eqs. (15·5) and (15·6) are not the most general expressions which could be used. They assume implicitly that both η and the density of the electrolyte are uniform through the electrolyte. At first sight, owing to the low compressibility of water, it might appear superfluous to question whether the density varies. But we are concerned with motion in the double-layer where, as we have seen in Section 2, very high fields can arise at which the water may be highly compressed owing to electrostriction.

The second term in Eq. (15·5), the so-called 'inertia-term' is very awkward, since it makes the equation non-linear in the velocity $\boldsymbol{v}$. In HENRY's analysis this term is dropped. To solve Eqs. (15·5) amd (15·6) it is now necessary to settle the values of ρ and ψ. HENRY makes the same assumption as SMOLUCHOWSKI's postulate 4. given above. For ρ the charge density in the double-layer of a symmetrically charged sphere in the state of thermodynamical equilibrium is taken; for ψ the potential in the double layer in the steady state is simply added to the potential which would result if the sphere carried no charge, but merely had a uniform conductivity different from that of the electrolyte. HENRY does not use the generalized POISSON-BOLTZMANN equation for the potential (Eq. (2·5)), but the simplified form (Eq. (2·8)). With these assumptions the equations are solvable. First, the divergence of Eq. (15·5) gives an equation for p only, not involving $\boldsymbol{v}$. Secondly, the curl of Eq. (15·5) gives an equation for curl $\boldsymbol{v}$, not involving p. From this and Eq. (2·3), the general solution for $\boldsymbol{v}$ is found. The boundary conditions at the surface of the particle give all the constants of integration except one, corresponding to the velocity at infinity, or in the experimental arrangement, to the rate of electrophoresis. This is finally evaluated from the condition that, since the sphere is

stationary in axes fixed with respect to its centre, the total force made up of the electrical term and a term arising from the fluid stresses at the surface must vanish.

Since all subsequent work on spherical particles may be regarded as generalizations of HENRY's calculation in the sense of removing one or more of the basic restrictions, it is convenient to summarize HENRY's simplifying assumptions.

1. Electrolyte incompressible.
2. Viscosity of electrolyte uniform.
3. The inertia term in the equation of motion can be dropped.
4. Potential is the sum of the equilibrium potential and the potential due to the external field.
5. The DEBYE-HÜCKEL approximation holds for the double layer contribution to the potential in assumption 4.
6. The conductivity of the material of the sphere is uniform.
7. No account is taken of the finite size of the ions in the electrolyte.
8. The Brownian motion of the solid particle is ignored in the calculations.

In what follows we shall indicate these assumptions by the symbol *A*.

The first simplification to receive attention was A4. KOMAGATA[94] pointed out that the motion of the electrolyte in the double layer will tend to alter the charge distribution. Owing to the finite mobility of ions, if a charge is suddenly created or destroyed in an electrolyte, a finite time is taken before the ions can redistribute themselves and come into statistical equilibrium once more. For the same reason, in electrophoresis we might expect the charge density in front of the moving particle to be *less* than its equilibrium value, and behind it to be greater. The asymmetry so produced in the charge distribution round the particle will clearly produce a field in the opposite sense to the applied field, and so tend to reduce the electrophoretic velocity. In addition to this asymmetry in the *diffuse* charge, changes may also occur in the charge density of the charge on the surface of the particle. Unfortunately, KOMAGATA's calculation was marred by several mistakes which rendered the final result valueless. To determine the distortion of the ionic atmosphere by the fluid flow, the disturbing effect of the solid particle on the flow was ignored. Also the effect of the disturbance of the spherical symmetry on the fluid stresses was taken into account, but the equally important direct effect on the surface charge was ignored.

The next attempt to deal with the ' relaxation effect ' as it was called, was made by HERMANS[95]. He confined attention to *thin* double layers and non-conducting particles, and assumed the distribution of charges on the particle itself retained spherical symmetry. He obtained the formula

$$U = \frac{\epsilon E \zeta}{4\pi \eta}\left[1 + \frac{e\,\zeta}{2kT} \cdot \frac{\sum_{i=1}^{s} n_i z_i^3}{\sum_{i=1}^{s} n_i z_i^2} - \frac{5\,\epsilon\,\zeta^2}{12\eta\pi kT} \cdot \frac{\sum_{i=1}^{s} n_i z_i^2 w_i^{-1}}{\sum_{i=1}^{s} n_i z_i^2}\right], \quad \ldots . (15{\cdot}7)$$

where n_i and z_i have the same meanings as in Eq. (2·5) and the w_i are the ionic mobilities. This formula was disturbing since, if it were true, both the SMOLUCHOWSKI and HENRY formulae would require very large corrections even for the lower values of ζ indicated by experiment.

But HERMAN'S analysis, like KOMAGATA'S, is open to serious criticism although on different grounds. The main objection is that although the electrostatic force on the particle due to the double layer distortion is taken into account, the equally important effects of the distortion on the fluid stresses were ignored. In other words, HERMAN'S method, if applied to the *primary* electrophoretic term, would simply have given STOKES' formula in which the force applied is simply the total charge multiplied by the external field. Hence formula (15·7) exaggerated the effect of relaxation.

Next we come to a very complicated investigation by OVERBEEK[96] in which the errors of KOMAGATA and HERMANS were avoided. OVERBEEK pointed out that, since the relaxation terms were of order ζ^2, for consistency, it was necessary to avoid A5 and use the exact solution for the steady state potential. Instead of using A4 it is necessary to solve the equations of motion and continuity, not only for the electrolyte, but for the ions as well. If uniformity of the ionic mobilities throughout the double layer is assumed, the equations for the ions are

$$\bar{\mathbf{u}}_i = \boldsymbol{v} - w_i\left(e\,z_i\,\text{grad}\,\psi + \frac{kT}{m_i}\,\text{grad}\,m_i\right), \quad \ldots . (15{\cdot}8)$$

$$\text{div}\, m_i\,\bar{\mathbf{u}}_i = 0 \quad \ldots . (15{\cdot}9)$$

$\bar{\mathbf{u}}_i$ denotes the mean velocity of the ions of type i at a point where the velocity is $\boldsymbol{v}$, the potential ψ and their concentration is m_i. The solution of Eqs. (15·5), (15·6), (15·8) and (15·9) is very difficult and a method of successive approximations is required. OVERBEEK assumed that the charge density on the surface of the particle remained unchanged; also, attention was confined to non-conducting particles. The calculation was carried as far as terms in ζ^3 and the result may be expressed by the formula

$$U = \frac{\epsilon\,\zeta E}{6\pi\,\eta}\left[f_1(b) - z^2\left(\frac{e\,\zeta}{kT}\right)^2 f_3(b) - \frac{(w_1 + w_2)\,\epsilon\,\mathbf{k}T}{12\,w_1\,w_2\,\pi\;\eta e^2}\left(\frac{e\,\zeta}{kT}\right)^2 f_4(b) + 0(\zeta^3)\right] \quad \ldots . (15{\cdot}10)$$

Only binary electrolytes were considered and the equation represents the result for a *symmetrical* electrolyte that is for an electrolyte containing equal numbers of positive and negative ions, each of valency z. The first term, involving $f_1(b)$, is the HENRY solution for the case λ equal to a half. The functions $f_3(b)$ and $f_4(b)$ were very complicated analytically, but exhibited fairly simple behaviour as functions of b. Both were zero for large values and small values of b and give simple maxima for b a little less than 10. Another formula was given for the unsymmetrical case. This contained a term in ζ^2, but all the functions in the term in ζ^3 were not completely evaluated. In this article it is impossible to discuss adequately the full implications of Eq. (15·10). Briefly, however, it may be said that relaxation is unimportant for very thin or very thick double layers, both descriptions of course being with reference to the particle radius. But, provided the zeta potential is sufficiently high, very serious discrepancies arise between Eq. (15·10) and HENRY's formula, Eq. (15·2), in the range of double layer thickness of about 1/20 to 2 times the particle radius. For example, OVERBEEK, using reasonable values for the mobilities, shows that for a value for $e\zeta/kT$ of 2, corresponding to a zeta potential of 50 millivolts, at room temperatures, and a 2-2 electrolyte, the correction to HENRY's formula amounts to a reduction of about 40 per cent. For ζ equal to 100 millivolts, the corrections become greater than the HENRY term, apparently making the electrophoretic velocity negative! This result is of course absurd. It merely indicates that for this particular value of ζ, Eq. (15·10), which of course represents just the first few terms of an infinite series in powers of ζ, is inadequate and the higher order terms are needed. Unfortunately, the analysis is too complicated to go much further although this would certainly be very desirable.

The same problem was examined independently by the present writer in a later paper (BOOTH[97]). It was assumed at the outset that the electrophoretic velocity is proportional to the field strength; in the usual experimental arrangement this is generally true. Also it was assumed that the surface charge remained unaltered. A general method was outlined for calculating the relation between U and Qe, the total charge on the particle, in power series form. It was shown that this relation was of the form

$$U = \frac{E\,e\,Q}{6\pi\,\eta\,a}\,X_1(b) + \frac{E}{6\pi\,\eta}\sum_{\nu=2}^{\infty} Q^{\nu}\,e^{2\nu-1}\,a^{-\nu}\,(\epsilon\,kT)^{1-\nu}\Big[X_{\nu}(b;\,q_2,\ldots q_{\nu}) + Y_{\nu}(b;\,q_2,\ldots q_{\nu}) + Z_{\nu}(b;\,q_2,\ldots q_{\nu},\,q_3^{*}\ldots q_{\nu}^{*})\Big] \quad \ldots.(15\cdot11)$$

This expression is easily converted into a power series in ζ. The first term in Eq. (15·11) is simply HENRY's formula. X_{ν} represents an additional term due to the *symmetrical* part of the field round the particle. Thus, if A5

only had been dropped in HENRY's calculation, we would obtain Eq. (15·11) with Y_ν and Z_ν equal to zero. Both Y_ν and Z_ν represent contributions to U arising from the disturbance of the symmetry of the double layer field round the particle, but Y_ν is the part of this contribution which is *independent* of the ionic mobilities. The calculation was not restricted to binary electrolytes and the q_m and $q_m{}^*$ coefficients were given by

$$q_m = \frac{\sum_{i=1}^{s} n_i z_i^{m+1}}{\sum_{i=1}^{s} n_i z_i^2} \; ; \; q_m{}^* = \frac{\epsilon kT \sum_{i=1}^{s} n_i w_i^{-1} z_i^{m-1}}{e^2 \pi \eta \sum_{i=1}^{s} n_i z_i^2} \quad \ldots . \; (15{\cdot}12)$$

The detailed estimates of the X_ν, Y_ν and Z_ν functions were carried out as far as the term $\nu = 4$ for *symmetrical* electrolytes; that is, electrolytes containing equal numbers of ions of the same charge, but with opposite sign. This means that the formula for U was correct to terms of order ζ^4 instead of those of order ζ^3 as in OVERBEEK's formula. The results for the terms of order ζ^3 agreed very closely with those of OVERBEEK. In view of the great complexity of the calculations and the independence of the methods this agreement is satisfactory.

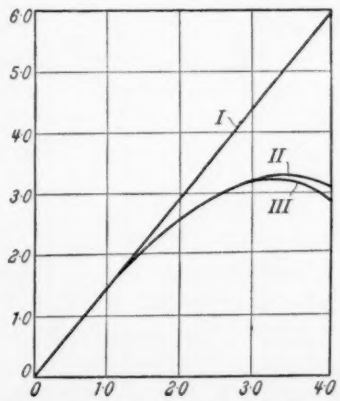

Figure 5. Dependence of electrophoretic velocity on the zeta potential for spherical particles with b equal to 6·3.
The ordinate is $6\pi\eta eU/E\epsilon kT$, and the abscissa $e\zeta/kT$.
Curve I represents HENRY's theory, and Curves II and III indicate the effect of taking relaxation into account.
In II terms of order ζ^4, and in III of order ζ^5, are dropped.

Some idea of the relative importance of the various terms of series (15·11) can be obtained from the accompanying figure. The corrections to HENRY's formula depend very much on the values of the constants q_m and $q_m{}^*$ and b. In Figure 5 we have plotted the quantity $6\pi\eta eU/E\epsilon kT$ as a function of $e\zeta/kT$ for pure water, and for b equal to 6·3. This corresponds to the point of *maximum* correction to HENRY's formula, if all other quantities are kept fixed and b only varied. The straight line through the origin is HENRY's formula. The effect of the term of order ζ^3 becomes appreciable for $e\zeta/kT > 1{\cdot}5$, and of the term of order ζ^4 when $e\zeta/kT > 4{\cdot}0$.

Since zeta potentials of more than 100 millivolts have been observed experimentally, the general conclusion to be drawn from these two memoirs is that HENRY's formula must only be applied with great circumspection if the double-layer thickness is of the same order of magnitude as the particle radius.

Before leaving the question of relaxation a simple proof by OVERBEEK[98] of the result of the more elaborate earlier calculation that for the term in ζ^3 the effect of relaxation vanishes for b large may be noted.

Now let us examine attempts to correct HENRY's formula in other ways. GORIN[99] treated electrophoresis, taking into account the finite size of the ions, A7. No attempt was made to deal with relaxation, but it was shown that the correction for the finite size of the ions becomes important when the ions are of significant size compared with the particle. This means, of course, that the correction is of importance for very small particles only. In these cases, however, it is natural to expect that the effect of the Brownian movement of the particle itself, A8, may cause considerable alteration in the theory of a method of treatment which assumes that the solid particle is in *steady* motion. The question of Brownian motion has been commented upon by OVERBEEK[100]. The article is mainly a review, but an interesting argument is put forward, based on the theories of conduction in ordinary electrolytes. It is shown that the Brownian motion may be neglected provided that the mobility of the particle (that is its velocity in the liquid when unit force is applied to it) is much smaller than the mobilities w_i of the ions. In general, this condition will be satisfied except for the smallest particles encountered; for example, some protein molecules.

The problem of electrophoresis when the conductivity of the particle is not uniform was examined independently by HENRY[101] and BOOTH[102]. The investigations were prompted by the observations of RUTGERS[86] on streaming potentials in glass tubes. To explain the results it seemed necessary to assume that the glass was coated with a thin layer of highly conducting material; or, in other words, that it exhibited high surface conductivity. The possible importance of the surface conductance effect in electrophoresis had been stressed earlier by BIKERMAN[103]. His quantitative results were not very useful however, since they only applied to particles in the form of long thin rods moving with their axes parallel to the direction of the field. It was shown by HENRY and BOOTH that the only effect of surface conductance in the theory of the electrophoresis of spheres was to change λ in Eq. (15·2) to λ_s, where

$$\lambda_s = \frac{a\,(\sigma_0 - \sigma) - 2\,\sigma_s}{a\,(\sigma + 2\,\sigma_0) + 2\,\sigma_s}\ , \qquad \ldots\ (15{\cdot}13)$$

and σ_s is the surface conductance. The importance of surface conductance

obviously depends on the relative magnitudes of $a\sigma$, $a\sigma_0$ and σ_s. For very thin double layers SMOLUCHOWSKI's formula is modified to

$$U = \frac{E\,e\,\zeta}{4\pi\,\eta} \quad \frac{a\,\sigma_0}{a\,\sigma_0 + \sigma_s} \quad . \qquad \ldots . (15{\cdot}14)$$

Using RUTGER's estimates for σ_s it was shown that for particles less than 10^{-3} cm in radius the effect of surface conductance might become of importance. In subsequent work, however, RUTGERS has given the opinion that his earlier estimates of the surface conductance were much too high. Consequently, surface conductivity may be of much less importance in electrophoresis than was at first thought.

The extension of HENRY's theory to include the 'inertia term' in the equation of motion was made by BOOTH[104]. If all the other basic assumptions of HENRY, except A3, are made it can be shown that the effect is to give a formula for U in powers of the field strength E in the form

$$U = \sum_{v=1}^{\infty} (\epsilon\,E\,\zeta)^v\,a^{v-1}\,\eta^{1-2v}\,W_v \quad , \qquad \ldots . (15{\cdot}15)$$

where the W_v are functions of b, and of the ratio of the particle conductivity to that of the electrolyte. A general method was given for calculating the W_v functions, but only the first *three* terms of series (15·15) were calculated explicitly. The first term of the series is again just HENRY's formula. The correction terms are only of importance for *large* values of b. For this case it was found that

$$U = \frac{\epsilon\,E\,(1+\lambda)\,\zeta}{6\pi\,\eta}\left[1 + \frac{67}{11{,}520\,\pi^2}\left(\frac{a\,\epsilon\,E\,\zeta\,(1+\lambda)}{\eta^2}\right)^2 + 0\,(\zeta\,E\,a)^3\right] \quad . \qquad \ldots . (15{\cdot}16)$$

Numerical estimates show that the correction is not likely to be large unless the applied field is of the order of a few thousand volts per cm.

Finally, we come to an analysis by BOOTH[105] on the effect of the modification of the *surface charge* on the particles during electrophoresis. In all previous work it has been assumed that the surface charge density remains during electrophoresis at its value when there is no applied field. But the surface charge cannot be regarded strictly as a permanent fixture of the surface. It arises through some form of chemical interaction with the solution; for example, through preferential adsorption of certain ions. Since the conditions near the surface may alter in electrophoresis, it does not follow necessarily that the surface charge density does remain unaltered. The analysis shows, however, that, provided the electrophoretic velocity is proportional to the field strength, then for non-conducting particles

HENRY's formula is still correct to the first power of ζ. For conducting particles this does not necessarily follow. Also, the relaxation terms of order ζ^2 and higher powers may, of course, be affected, but calculations on these are not yet complete.

(*b*) *Electrophoresis of non-spherical particles*—Here the theory is much less complete than for spheres. For particles for which the double layer thickness is everywhere very small compared with the radius of curvature of the liquid-solid interface, SMOLUCHOWSKI's formula can be applied. HENRY[93] showed that for long cylinders aligned *parallel* to the field SMOLUCHOWSKI's formula holds quite generally, whatever the conductivity of the solid or the double layer thickness. For a non-conducting cylinder, with its axis *perpendicular* to the field, he showed that the right-hand side of Eq. (15·1) should be divided by two if the double layer thickness is very great compared with the radius. The mobility, when the double layer thickness is neither very large nor very small compared with the radius, can be calculated fairly easily from HENRY's analysis using numerical integration (ABRAMSON, GORIN and MOYER[106]).

(*c*) *Electrophoresis of liquids*—Little detailed work has been done on the electrophoresis of liquid droplets. FRUMKIN[107] has discussed the electrophoresis and sedimentation of mercury drops. He emphasized that the behaviour would be quite different from that of solid particles owing to the flow of liquid inside the drop. He considered that the variation of the interfacial tension over the surface of the drop was of great importance, giving rise to much higher rates of electrophoresis than for solid particles of the same radius and zeta-potential.

BOOTH[108] has developed a general theory of the electrophoresis of spherical drops of fluid, corresponding closely to that of HENRY for spherical solid particles. With regard to the potential in the electrolyte the same assumptions were made as in HENRY's calculation, and relaxation effects were neglected. For the charge distribution in the drop itself three cases were examined; first a uniform *surface* distribution of charge concentrated on the interface; secondly a uniform *volume* distribution of charge; and finally for a double layer charge in the drop. It was assumed that the surface tension remained uniform over the interface. The analysis differs from that for solids in that account must be taken of the motion of fluid within the drop. Also the boundary conditions at the interface are different. The various formulae for the different cases show wide differences, and also are very different from the corresponding formulae for solids.

16. *Electroviscous effect*

This concerns the viscous properties of sols but it is a much less striking effect than either thixotropy or dilatancy. The ordinary macroscopic

viscosity of a sol containing uncharged particles is slightly greater than that of the pure solvent, as one might expect, since part of the medium is replaced by rigid bodies. If in addition the sol particles are charged the effect of the ionic double-layer round them is to give a further small increase in the overall viscosity. This increase is known as the electro-viscous effect. Two main theories have been developed, the first for suspensions of spherical particles and the second for solutions containing particles in the form of long chains. We shall accordingly consider the two separately.

(*a*) *Solid spherical particles*—The first theoretical investigation of the viscosity of suspensions was made by EINSTEIN[109] in 1906. He showed that the effective viscosity η of a suspension of equal solid spheres uniformly distributed in a liquid of viscosity η_0 is given by

$$\eta = \eta_0 (1 + 2{\cdot}5\,\phi) \quad , \qquad \ldots . (16{\cdot}1)$$

where ϕ is the volume ratio of the solid to the whole suspension. The formula is only valid when ϕ is small compared with unity, and it is easy to see that terms of higher powers of ϕ should be included. The extension of the formula is, however, very difficult, but GUTH and SIMHA[110] in 1936 gave

$$\eta = \eta_0 (1 + 2{\cdot}5\,\phi + 7{\cdot}8\,\phi^2 + \ ) \quad , \qquad \ldots . (16{\cdot}2)$$

SMOLUCHOWSKI[111] pointed out in 1916 that if the spherical particles were charged then one would expect the viscosity to be increased slightly. He gave, without proof, the relation

$$\eta = \eta_0 \left[1 + 2.5\,\phi \left(1 + \frac{1}{\sigma\,\eta_0\,a^2} \left(\frac{\zeta\,\epsilon}{2\pi} \right)^2 \right) \right] , \qquad \ldots . (16{\cdot}3)$$

where ζ is the zeta-potential, ϵ the dielectric constant of the liquid of the suspension, σ the specific conductivity of the electrolyte, and a the radius of the particles. The formula holds with the same restriction as the EINSTEIN formula ($\phi \ll 1$), and only provided that the double layer thickness is small compared with the radius.

Later, KRASNY-ERGEN[112] furnished a derivation of Eq. (16·3). His result showed that formula (16·3) required a numerical alteration, the last term being multiplied by 1·5.

A considerable amount of experimental work has been done on the effect. This confirms many of the qualitative predictions of Eq. (16·3); for example, increase of viscosity with decrease of a and of the conductivity. But quantitatively the agreement is not very satisfactory since the formula seems to indicate far too great an increase in η. Thus, some observations on egg albumen by BULL[113] gave calculated values nearly 150 times too large.

Any experimental check of Eq. (16·3) is very difficult, however, owing to the smallness of the effect when the conditions necessary for the validity of the formula are imposed. In the first place, the condition that ϕ must be very small compared with 1 means that very dilute sols, with only slight difference in viscosity from that of the pure liquid, can be used. With the usual values for the zeta potential the electrical term of Eq. (16·3) is small compared with 1, unless both σ and a are small. Hence, the electrolyte must be dilute and the particles small. Taken together, these imply that the double-layer thickness may have to be *large* compared with the radius. But the formula only applies if the thickness is *small.*

An attempt to provide a theory of the electroviscous effect in which no restriction was imposed on the double-layer thickness has been made by BOOTH[114]. The general principle was to derive appropriate solutions of the equations of motion and of continuity of both ions and liquid (Eqs. (15·5), (15·6), (15·8) and (15·9)), by the method of successive approximations used in the analysis of electrophoresis. The technique of calculating the effective viscosity of the suspension was based on a method due to FRÖHLICH and SACH[115]. We shall not give details of the final solution, but it was shown that even for a *thin* double-layer KRASNY-ERGEN'S formula requires correction. The electroviscous term in Eq. (16·3) should be multiplied by a very complicated function of κa which tends to zero for large values but increases as κa becomes small; κ is defined by Eq. (2·9). Better agreement with experiment was obtained using the amended theory than with KRASNY-ERGEN'S equation, but the concordance still left much to be desired. The difficulty in comparing experiment with theory for very small particles arises from the difficulty of knowing whether the effective radius itself remains unaltered as the concentration of electrolyte is varied. The usual method of estimating the electroviscous contribution as a function of ionic concentration is to measure the difference between the viscosity at the isoelectric point and at various other concentrations. Owing to hydration, however, the size may depend on the concentration of electrolyte. It would appear that satisfactory tests of the theory must await the development of far more sensitive methods of viscosity measurement. It might then be possible to use much larger particles of known spherical form and radius.

(*b*) *Long chain molecules*—A large number of hydrophilic colloids, like starch and agar-agar, show a strong electroviscous effect. The results on these materials have often been interpreted in terms of the SMOLUCHOWSKI formula. HERMANS and OVERBEEK[116] have pointed out, however, that, since it is very probable that these colloidal particles in solution approximate to loosely linked chains rather than solid spheres, little significance can be attached to such interpretations. They suggest an entirely different origin for the reduction of viscosity by electrolytes in these cases. The long chain

molecule is pictured as a random coil moving through the liquid owing to Brownian motion. If the concentration of electrolyte is low the charges distributed along the molecule will repel each other and so tend to straighten the chain. But increase of electrolyte concentration will reduce the mutual repulsion of the charges owing to double layer screening, and so the chain or coil becomes more compact. Consequently, it interferes less with the fluid motion and so the apparent viscosity is reduced.

The detailed analysis is based on work originally developed to explain the properties of rubber. The chain is pictured as a series of N equal, randomly linked elements each of length s. If F is the free energy of the coil due to its charges and the accompanying double layer, then the mean square of the distance h between its two end points is given by

$$\overline{h^2} = \frac{\int_0^\infty dh\, h^4 \exp\left(-\frac{3h^2}{2Ns^2} - \frac{F}{kT}\right)}{\int_0^\infty dh\, h^2 \exp\left(-\frac{3h^2}{2Ns^2} - \frac{F}{kT}\right)} . \qquad \ldots . (16{\cdot}4)$$

The next question is how to relate this quantity to the viscous properties. Obviously the greater $\overline{h^2}$ the greater the increase in viscosity, but this qualitative idea must be put in precise mathematical form. Fortunately, this problem had already been solved by DEBYE[117]. For a 'free-drained' polymer coil, that is, one in which the flow past each segment can be assumed not to affect that of its neighbours, the effective viscosity η is given by

$$\eta = \eta_0 + \frac{nf}{6} \sum_K \overline{r_K^2} . \qquad \ldots . (16{\cdot}5)$$

η_0 is the viscosity of the pure solvent, n the number of chains per unit volume, f is the 'friction constant' of each segment, and $\overline{r_K^2}$ the statistical average of the square of the distance of the centres of the segments, distinguished by the suffix K from the centre of gravity of the whole polymer chain. Suppose we now define a quantity R by the equation

$$R^2 = \frac{5}{3N} \sum \overline{r_K^2} . \qquad \ldots . (16{\cdot}6)$$

If all the polymer segments were distributed uniformly through a sphere, then the sphere would be of radius R. For in this case we would have

$$\overline{r^2} = \frac{\int_0^R 4\pi r^4 \, dr}{\int_0^R 4\pi r^2 \, dr} = \frac{3}{5} R^2 \, . \qquad \ldots . (16{\cdot}7)$$

Hence we may regard R as the ' effective radius ' of the coil. Further, if the rough approximation is made that the friction constant can be replaced by the STOKES value $3 \pi \eta_0 s$, which implies that we treat each segment as a small sphere, from Eqs. (16·5) and (16·6) we find

$$\frac{\eta - \eta_0}{\eta_0} = \frac{3 \pi n s N R^2}{10} \, . \qquad \ldots . (16{\cdot}8)$$

The next step is the calculation of F, the electrical free energy, in terms of R. This is rather complicated, but the authors were finally able to give a simple interpolation formula

$$F = \frac{3 (Ze)^2}{5 \epsilon R} (1 + 0{\cdot}6 \kappa R + 0{\cdot}4 (\kappa R)^2)^{-1} \, , \ldots . (16{\cdot}9)$$

where Ze is the total charge on the chain and ϵ the dielectric constant of the liquid. In principle the problem is now solved. For a chosen R, F can be found from Eq. (16·9). Substitution in Eq. (16·4) gives the statistical average of h^2. But this can be related to R and the calculation will be consistent provided R was correctly chosen originally. HERMANS and OVERBEEK showed that they could reproduce viscosity curves very much like the experimental ones, but for quantitative comparison the charge and dimensions of the molecule would be required.

Later, the theory was tested quantitatively by ARNOLD and OVERBEEK[118] in a very ingenious way. Solutions of polymethacrylic acid were partially neutralized to varying extents and the viscosities and *p*H values of the resulting solutions measured. In an earlier paper OVERBEEK[119] had given a calculation of the dissociation constants of polymer long chain acids as a function of the effective radius R and the charge. The calculation followed closely that of Eq. (16·9). From this theory and the *p*H values, the effective radius R was calculated; the charges on the molecules were calculated from the degree of polymerisation. Other estimates of R were then obtained from the viscosity measurements using DEBYE's formula (Eq. (16.5)). Reasonable agreement between the two sets of values for R was obtained for *low* degrees of ionisation. For higher degrees of ionisation a better fit was obtained by using, for the calculation of F in Eq. (16·9), a uniform *surface* distribution of charge at the mean square radius of the coil. But

when the degree of dissociation became greater than 0·2 there was a steep rise in the observed viscosity. This was not paralleled by a corresponding sudden rise in the effective radius calculated from the *p*H observations. ARNOLD and OVERBEEK point out that this is probably due to the breakdown of their theory in two respects. In the statistical part of their theory they assumed, in the correlation of R and h, that the effective radius R was small compared with the total length of the coil. But if the repulsive forces between the various segments are sufficiently strong the whole molecule may be straightened out sufficiently for R and the total length to be of the same order of magnitude. Further, in the calculation of the free energy F due to the charges and their double layers they invoked the DEBYE-HÜCKEL approximation which may not be adequate. A calculation based on the general POISSON-BOLTZMANN equation (Eq. (2·5)) would be more satisfactory.

17. *Electrical effects due to sound waves*

DEBYE[120] was the first to point out that when plane sound waves traverse an ordinary electrolyte small potential differences might be set up between suitable points in the wave train owing to differences between the masses of the ions. His object was to provide a theory of some method which might furnish information on ionic masses in solution. Such information is very difficult to obtain since, owing to the small dimensions of ordinary ions, in all the usual transport processes (for example conduction or viscosity) inertial forces may be ignored in comparison with viscous forces. Owing to the smallness of the effect, however, DEBYE'S suggestion has not proved very fruitful and its existence has only recently been detected experimentally by YEAGER, BUGOSH, HOVORKA and MCCARTHY[121].

HERMANS[122] pointed out that the same effect was to be expected in colloidal solutions since the particles may be regarded as merely abnormally large and highly charged ions. Following DEBYE'S calculation he made the assumption that the region of hydrodynamic disturbance due to the particle was small in volume compared with the double-layer region around the particle. This assumption is probably adequate for ordinary ions; but colloidal particles are generally so much larger than ions that the theory has little application. The potential differences in the wave train are, in fact, set up by the distortion of the double layers surrounding the colloidal particles in exactly the same manner as the sedimentation field in steady sedimentation and the relaxation field in electrophoresis. If the colloidal particles were of the same density and possessed the same stress-strain properties as the liquid the sound waves would pass through the suspension in exactly the same manner as for the pure electrolyte. In fact, the particles will possess rigidity, a different compressibility from the liquid, and will in general be denser. Consequently, each particle will

modify the fluid motion to some extent in its neighbourhood. Now the ions forming the double layer around the particle will tend to move with the liquid; consequently the charge density in the double layer is modified. The net effect of this variation of charge density is to give, outside the double-layer, a potential variation equivalent to that due to a dipole situated at the centre of the particle, its axis lying in the direction of propagation of the sound, and its moment varying harmonically with the frequency of the sound waves. If we now consider the suspension as a whole, the distribution of dipoles will set up potential differences between different positions in the wave train, in the same way as potential differences arise in polarized dielectrics.

Using this general model, HERMANS[123] in a second paper calculated the amplitude of the potential differences set up in a uniform distribution of spherical particles, all carrying the same charge and possessing the same zeta potential.

The same problem was examined by ENDERBY[124], but in his analysis no restriction was imposed on the relative dimensions of the double layer thickness and the particle radius. It was assumed that the wavelength of the sound waves was large compared with the size of the colloidal particles, and also that the mean separation of neighbours was much greater than the double-layer thickness. The first condition allows the rigidity and compressibility of the particles to be ignored so that the velocity disturbance is due solely to their inertia. The second simplifying condition allows the double-layer field of each particle to be considered separately and mutual interactions ignored. A general expression was derived from the potential differences set up in the case when

$$f \ll \kappa^2 w^1 kT \quad , \qquad \ldots . (17{\cdot}1)$$

where f is the frequency of the sound waves, w^1 the mobility of the slowest ions in the electrolyte, k BOLTZMANN'S constant, T the temperature, and κ^{-1} the double-layer thickness. The general expression for the potential difference Φ over a half wavelength is given in Eq. (3·27) of the paper. Owing to its complexity, we shall not give the formula in its exact form in this account. With a number of reasonable approximations it was possible to show that the general expression reduced to a fairly simple result provided the frequency was not too high. The value of Φ correct to the first power of ζ was

$$\Phi = \frac{A\, mq\, \lambda f a^2 \zeta}{72\, \eta\, kT}\left(1 - \frac{\rho_0}{\rho_1}\right)\frac{B(b)}{b^2} \qquad \ldots . (17{\cdot}2)$$

where A is the velocity amplitude of the sound waves in the liquid;
m is the total mass of the colloidal particles in unit volume;

λ is the wavelength of the sound-waves in the solution;
a is the radius of the particles;
ρ_1 is the density of the material of the particles;
ρ_0 is the density of the electrolyte

q and b are defined by the equations

$$q = \frac{\sum_{i=1}^{s} n_i z_i^2 w_i^{-1}}{\sum_{i=1}^{s} n_i z_i^2}, \quad b = \kappa a \qquad \ldots . (17{\cdot}3)$$

where n_i, z_i and w_i are the concentrations (outside the double-layers), valency, and mobilities respectively of the various ions present. B (b) is an increasing function of b, but owing to the factor b^{-2}, Φ decreases to zero as b becomes large. A careful comparison of theory and experiment would require the exact formula, but Eq. (17·2) is adequate for good estimates of orders of magnitude. Rough calculations indicate values of a few millivolts for silver iodide sols in very dilute electrolytes. As the concentration of electrolyte is increased the potential rapidly falls to very low values. These estimates are in much better agreement with experimental values than those of HERMANS, although at the present stage the experimental results are too scanty to say definitely whether there is close agreement.

The calculation of the potential when condition (17·1) no longer applies, a state of affairs which might obtain for comparatively thick double-layers, is very difficult. However, it was possible to show that, provided the ionic mobilities are all identical and a certain boundary condition applies at the surface, the formula (17·2) for the potential is simply multiplied by a factor β, where

$$\beta = \frac{\kappa^2}{\left[\kappa^4 + \left(\frac{f}{2\pi w kT}\right)^2\right]^{\frac{1}{2}}} \qquad \ldots . (17{\cdot}4)$$

In another paper however, (BOOTH and ENDERBY[125]) the problem was solved for the general case when the ionic mobilities are not all equal. It is not possible to give a simple formula for Φ except in the case of a simple binary electrolyte.

18. *Future work*

Our account of recent work is now complete. In conclusion it might be of interest to make a few observations on future developments. It is, of course, very risky to forecast the future direction any theory will take, but a few indications at least of desirable extensions can be given.

In the first place it would be very useful to have some new method of treating electrolytic solutions quite generally, but without the defects of the DEBYE-HÜCKEL theory; in particular some theory of concentrated solutions of electrolytes is badly needed. In the field of colloidal stability, possible improvements in detail seem likely. The effect of particle shape, ionic image forces and the finite sizes of ions might be studied. But it does not seem likely that major changes in the existing theory would result. More scope for development would seem to lie in the study of the elastic properties of sols and gels; for example, the basic cause of thixotropic behaviour is still an open question.

In the theory of electrokinetic effects, two important problems still await solution. There is as yet no adequate analysis of the electrophoresis of particles of non-spherical form, except in the limiting case when the SMOLUCHOWSKI formula can be used. Also there is no general theory of the electrophoresis of *suspensions* of particles in finite volumes of liquid as distinct from the electrophoresis of single particles in an infinite fluid, the arrangement usually assumed in calculations.

The greatest need however in the study of electrokinetic effects would seem to be a careful comparison of existing theory and experimental work. The concordance between values of the zeta potential for the same systems obtained by different methods leaves much to be desired. It would be very useful to know whether this situation is due to accidental circumstances such as surface impurities, or the fundamental inadequacy of the theory as it stands at present.

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MICROSPECTROMETRY OF LIVING AND FIXED CELLS

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INTRODUCTION

THE chemical composition of cellular structures and the changes that occur in them during growth, cell division and differentiation may be studied by a variety of methods. One procedure has been to isolate material in bulk from large numbers of cells or separated cell constituents. This material is then analysed by the many chemical and physical techniques which are available. Detailed information on the composition and structure of many compounds of biological origin, such as the proteins and nucleic acids, is now available, together with data on the chemical composition of isolated cell components such as nuclei, mitochondria and microsomes. These methods involve a long series of steps beginning with cell disruption, and at each stage there is the danger of interchange between the various components, or the loss of important chemical constituents. It is sometimes difficult therefore to determine the original site of the material extracted, which may itself bear little relation, either chemically or physically, to that originally present in the living cell. There is another difficulty inherent in the bulk biochemical approach. Large amounts of tissue are required which may contain many different cell types; the results obtained are only average values. At the other extreme, where measurements are made on the single cell, localization can be more precise, but the methods of analysis are limited mainly to the optical ones. By a combination of the techniques of microscopy and spectrophotometry it is possible in principle, to apply absorption, fluorescence and Raman spectroscopy to the individual cell and its components. So far, the absorption in the visible and ultra-violet regions of naturally occurring chemical compounds, and the products of certain colour reactions have been mainly investigated, and it is with these measurements that this article is concerned.

The advantages of the first ultraviolet microscope (KÖHLER[1], 1904) were described as twofold. First, the resolving power which varies inversely as the wavelength was increased. Second, ultra-violet photomicrographs of living cells showed much more detail and contrast especially in the chromosomes, than photographs taken with visible light; this was later to prove of the greatest significance in cytochemistry. The main emphasis for the next

thirty years was upon the investigation of fine structure and the morphological study of living or unstained material. For example, the structure of ectromelia virus was studied by BARNARD and ELFORD[2] (1931), that of living chromosomes in Grasshopper spermatocytes by LUCAS and STARK[3] (1931), and mitosis in tissue culture cells by WYCKOFF, EBELING and TER LOUW[4] (1932). The last workers attempted to minimise the lethal action of the ultraviolet light by using a small magnification on the photographic film.

During this period it was shown that the nucleic acid from yeast (DHÉRÉ[5], 1906) and thymus nucleic acid (HEYROTH and LOOFBOUROW[6], 1931) had similar high absorptions with maxima at 260 mμ, due to the purine and pyrimidine bases. However, despite the fact that WYCKOFF *et al.*[4] (1932) had commented on the similarity between photomicrographs at 257 mμ and those of Feulgen stained preparations, no serious attempt was made to correlate morphological features and chemical composition. (See the discussion to a paper by WYCKOFF[7], 1934). It remained for CASPERSSON[8] (1936) to demonstrate the full power of microspectrometry as a cytochemical method. He showed that the absorption of certain cell structures was in good agreement with that found for the nucleic acids, and pointed out the extreme sensitivity of the method whereby quantities as small as 10^{-12} gm could be measured. His paper was the forerunner of a long series dealing with protein metabolism and the nucleic acids (for a full bibliography see CASPERSSON[9], 1950).

Chemical analysis by absorption spectrophotometry is accomplished by applying the LAMBERT-BEER law which states that for any monochromatic wavelength

$$E = \log_{10} \frac{I_o}{I_T} = kcd \qquad \ldots . (1)$$

where E is the extinction, I_o the incident intensity, I_T the transmitted intensity, c the concentration, k the extinction coefficient, and d the thickness of the specimen. From the shape of the absorption curve it may be possible to identify the absorbing substance by a comparison with the spectral absorption of known isolated compounds. Substances can be detected in the cell by this method, provided they are present in sufficient amounts and with high enough extinction coefficients to absorb a measurable fraction of the incident energy. Examples of some important absorption curves, those for a nucleic acid and two proteins, are illustrated in Figure 1a; the absorption coefficients of the proteins are relatively small in the region of the spectrum which can be conveniently studied, and only those containing the aromatic amino acids tyrosine, tryptophane and phenyl alanine (Figure 1b) have specific absorption in this region. The extinction (E), usually measured at the absorption maximum, is proportional to the

amount of absorbing material per unit area; from measurements of the cell geometry the total amount and the concentration may be calculated.

On the macro-scale absorption spectrophotometry is a highly accurate and well understood method. On the micro-scale, the nature of the biolo-

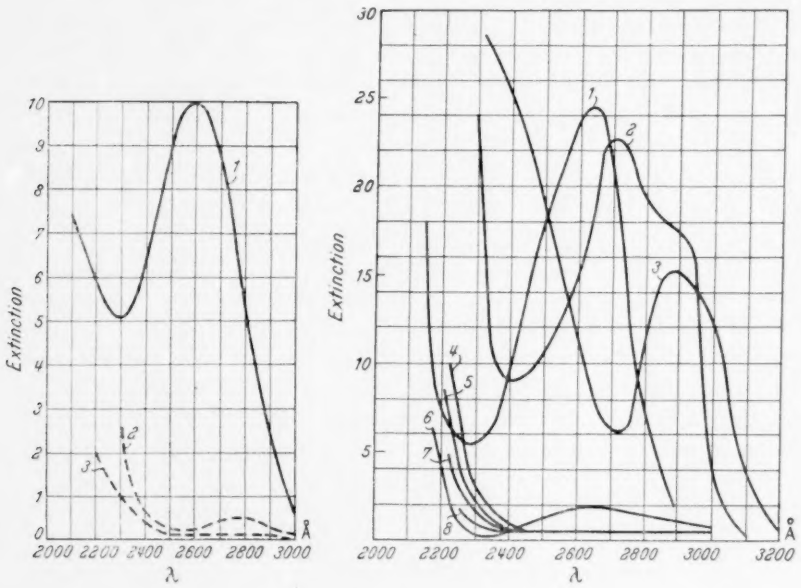


Figure 1(*a*). Comparison between absorption of nucleic acids and proteins in similar concentrations, showing (1) thymonucleic acid, 1 cm and 0.5 per cent; (2) serum albumin 0.5 per cent; (3) protamine sulphate 5 per cent.
(*b*) Shows the absorption spectra of (1) adenine, (2) tryptophane, (3) tyrosine, (4) histidine, (5) arginine, (6) phenylalanine, (7) leucylglycin, (8) proline. (Adenine $\frac{1}{3}$ per cent and others 1 per cent, 1 cm thickness).
(From T. CASPERSSON[9] by permission *W. W. Norton & Co.*, New York.)

gical material, the complexity of staining reactions, and the optical conditions in the microscope give rise to extra difficulties and errors; only by paying special attention to them and realizing the limitations of the techniques may valid results be obtained.

INSTRUMENTS AND TECHNIQUES

The measurement of cellular absorption requires the proper choice and combination of light source, monochromator or spectrograph, microscope, and recording device. This section will briefly describe microscope objectives, especially those for use in the ultraviolet, the various ways in which the above components can be arranged and used for microspectrographic analysis, and the particular advantages of the different optical

systems. Microspectrographic investigations up to 1948 have been reviewed by SCOTT and SINSHEIMER[10] and more recent developments by LOOFBOUROW[11] and by MELLORS[12].

Microscope objectives

Most microspectrographic investigations, including the classical researches by CASPERSSON, have been made with monochromat objectives of fused quartz first described by KÖHLER and VON ROHR[13] and manufactured by *Zeiss*. It was found that although designed for one wavelength, namely 275 mμ, they could be used at other wavelengths in the range 230—430 mμ with narrow bandwidths of radiation only, ($\sim$ 0·5 mμ[14]), but with some deterioration in the quality of the image. An attendant difficulty in their use is that of focusing at the series of wavelengths required for a complete absorption curve. One method is to focus the objective in monochromatic visible light and then move the microscope fine adjustment through predetermined amounts. Alternatively a fluorescent screen may be used, but there is then the danger of photolysing the specimen. In the ultraviolet microscope described by BARNARD and WELCH[15] focus was obtained with a visible apochromatic objective, which could then be replaced with considerable mechanical precision ($\sim$ 0·1 μ) by the parfocal ultraviolet objective; this gives precise focus at a single wavelength only. DAVIES and WILKINS[16] have described a simple alternative method in which a single glass lens is placed behind the ultraviolet objective. Focus is found in visible light and when the lens is removed the objective is in focus for one ultraviolet wavelength. Owing to the difficulty of focusing, the use of monochromat objectives is confined mainly to high resolution photomicrography[17–20]. For this purpose the extremely well designed high aperture immersion monochromats now manufactured by *Cooke, Troughton and Simms*, or the equivalent *Zeiss* designs, are superior to any of the available achromatic objectives.

Achromatism resulting in a simplification of technique has been achieved in two ways. Quartz and lithium fluoride lenses are suitably combined, or use is made of reflection from curved surfaces, an inherently achromatic process. Achromatic refracting objectives have been designed by JOHNSON[21] and FOSTER and THIEL[22] and a high aperture (N.A. 1·24) design by BRACEY[23] has recently been manufactured by *R. & J. Beck* for use in our laboratory. The use of mirror systems in the microscope optics has greatly extended both the range of complete achromatism and the light transmission. It should now be possible to extend the scope of microspectrometry to include the vacuum ultraviolet. Infra-red microspectrometry has already been developed by BARER, COLE and THOMPSON[25], BLOUT, BIRD and GREY[24], and FRASER[26]. Absorption coefficients are not so high as in the ultraviolet and the resolving power of the microscope is

inherently lower; for these reasons most single cells are too small to be analysed. Infra-red microspectrometric analysis of biological compounds has been reviewed by FRASER (this volume).

The most highly developed form of reflecting microscope, consisting of an aspheric mirror pair arranged in a SCHWARZSCHILD[27] system, has been designed and constructed by BURCH[28,29]. Other objectives using only spherical mirrors, which are much more easily manufactured, have been described by many workers including MAKSUTOV[30], BRUMBERG[31], GREY[32–35], SEEDS, WILKINS and NORRIS[36–37]. Systems in which refracting components have been added to raise numerical aperture and correct for the aberrations of the spherical mirror pair, which increase rapidly when the numerical aperture is raised above 0·5, have been described by GREY[32–35]; there is then a sacrifice in the range of achromatism and the light transmission is somewhat reduced. The numerical aperture of the mirror pairs can be increased by the addition of a hemispherical immersion component, by a factor equal to its refractive index ($\sim$ 1·5 for fused quartz). An objective designed on the Amici principle, by which the numerical aperture is increased by the square of the refractive index of the added immersion component, has been described by NORRIS and WILKINS[38]; this objective is not achromatic. A disadvantage of the reflecting objectives is obscuration by the central on-axis mirror of part of the light beam from the specimen; this results in light being thrown out of the central Airy disc diffraction image of a point object into the adjacent rings. The effect of central obstruction has been studied in telescopes by DALL[125] (see also DUNHAM[39]) who finds visually that only when it is less than 20 per cent is the effect on the image negligible. An important advantage of the BURCH system is that the linear obstruction ratio is low, about 0·14. Reflecting objectives require carefully positioned stops[37] to prevent unwanted light flooding the image.

Combination of microscope and monochromator or spectrograph

Two methods of illumination are in use; either the monochromator precedes the microscope, Figure 2a, b (Method I) or a spectrograph is placed after the microscope, Figure 2c (Method II). Each arrangement has its own particular advantages depending on the type of problem to be investigated.

Method I

In the simplest form light filters are used and the microscope illuminated according to KÖHLER (HARDY and PERRIN[40], p. 507). This system works best in the visible range where narrow bandwidths can be isolated by filters relying on selective absorption, selective scattering (Christiansen filters) or interference. An apparatus for the photoelectric measurement of

stained cells has been described by POLLISTER and RIS[41] (see also POLLISTER and MOSES[42]).

For several reasons it is desirable to use a monochromator in the ultraviolet, and double monochromators can be used with advantage to exclude scattered radiation. In the usual Köhler system for photographic recording (Figure 2a) there is no variation in wavelength across the field stop. The

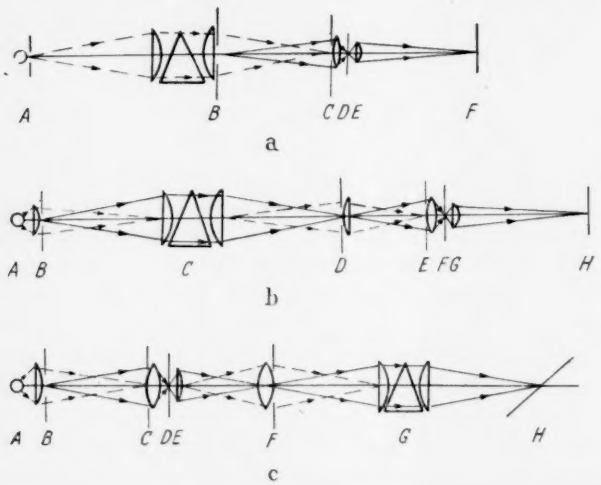


Figure 2. Schematic arrangements of light source, monochromator or spectrograph, microscope, and radiation detector; for simplicity of representation only single lenses are drawn.

(*a*) The source at *A*, the entrance slit of the monochromator, is imaged by the monochromator on to the *aperture stop* behind the condenser at *C*, which in turn forms an image of the *field stop B* on the specimen plane at *D*; the objective *E* images *D* on to the receiver at *F*.

(*b*) The light source at *A* is imaged by the lens adjacent to the entrance slit *B* of the monochromator on to the prism at *C*. The lens *D* at the exit slit (*field stop*) images the prism on to the *aperture stop* at *E*. The condenser forms an image of *D* on the specimen plane at *F*.

(*c*) The source at *A* is imaged by the lens *B* on to the *aperture stop* at *C*, while the condenser forms an image of the *field stop B* on the specimen plane *D*, which is then imaged by the objective *E* on to the lens *F* adjacent to the entrance slit of the spectrograph. The lens *F* forms an image of *E* on the prism at *G* and the spectrograph images *F* on to the detector at *H*.

optical system shown in Figure 2b, which also incorporates Köhler illumination, is suitable for photomicrography with line sources only; the input slit of the monochromator is opened until the spectral lines at the exit slit (the field stop) are contiguous. It is also used for the photoelectric recording of absorption spectra in small areas of the cell with narrow bandwidths of radiation from a continuous light source.

It is convenient to choose a monochromator with a low aperture telescope lens (of the order f/30) to match that of the microscope. When it has not

been specially designed, in the first system the required exit slit determining the bandwidth may not be equal in size to the diameter of the condenser lens or, in the second system, the angle subtended at the exit slit by the monochromator lens may not be equal to that subtended by the condenser lens; in each case two extra lenses or mirrors are required between the exit slit of the monochromator and the condenser. The general equation[11] describing any system is

$$\frac{f_1}{d_1} \div \frac{f_2}{d_2} = \frac{d_s}{d_f}\left(1 + \frac{1}{m}\right) \qquad \ldots . (2)$$

where $\frac{f_1}{d_1}$ and $\frac{f_2}{d_2}$ are the aperture ratios of the monochromator telescope and microscope objective respectively; d_s the monochromator slit width, d_f the width of the illuminated portion of the microscope field and m the magnification.

It will be recalled that for any fixed bandwidth the brightness (radiant power per unit solid angle per unit area) in the object plane, apart from transmission losses, is equal to that of the source; also the angular cone of illumination through the microscope is determined by the condenser aperture. For this reason increasing monochromator size, either by scaling it up or increasing the number of prisms, does not alter photographic exposure time (determined by radiant power per unit area) but increases field size. Under similar conditions photoelectric response (determined by total radiant power) is unaltered, since the area of object to be analysed is fixed; photoelectric response can only be increased at the expense of using greater bandwidths of radiation. These considerations emphasise the need for choosing light sources with the highest possible brightness if photographic exposure times are to be conveniently short, and adequate photoelectric response obtained for absorption measurements on small areas.

Quantitative methods were first introduced in ultraviolet microscopy by CASPERSSON[8,43] and the principles of measurement are best outlined by referring to his apparatus, which employed quartz optics, together with KÖHLER[44] rotating spark gap and low, medium, or high pressure water-cooled mercury arcs as sources. The procedure for photographic photometry consists in comparing the photographic density of points in the image of the object with that of an adjacent clear field. This requires even illumination across the field which may be difficult to attain; it is then necessary to make preliminary measurements on a blank field. The relationship between light exposure and photographic density must be known at each wavelength. More satisfactory calibration methods than that originally described by CASPERSSON are the quartz step-wedge (UBER[45]) and the rotating sector (COLE and BRACKETT[44]) whose image or shadow should be

placed as close as possible to the measured area to avoid error from variations in photographic plate sensitivity. A step-wedge of rhodium evaporated on to quartz is manufactured by *A. Hilger*; the light transmission is slightly dependent on wavelength, but it is easily calibrated by means of a Beckman spectrophotometer. The advantage of the rotating sector is its wavelength independence, but interference with an intermittent light source must be avoided. THORELL[46] has discussed the errors arising from intermittency effects and failure of reciprocity law in photographic materials when a sector is used, and he concludes that under appropriate conditions these are small.

The system of photoelectric photometry described by CASPERSSON[43] used as detector a photocell and electrometer together with an auxiliary light beam and second photocell to correct for variation in source intensity. The principles of measurement are as follows : a small central part of the field is illuminated and this is projected on to the aperture of the photocell; this aperture corresponds to a field diamater of about 0·2 μ. The radiant flux through an object area is compared with that through a clear region of the field by a null method using a rotating sector of variable aperture to reduce the light flux. The method requires that the object shall be accurately re-positioned for measurements at other wavelengths; this is accomplished by a special moving stage (re-alignable to $\sim 1/20\,\mu$). Errors from uneven illumination of field are thus obviated.

Recent developments in instruments employing achromatic objectives include a continuous recording visible and ultraviolet microspectrophotometer built by SINSHEIMER[47] and a colour translating microscope designed by LAND *et al.*[48]. In the former system an R.C.A. I.P. 28 cooled photomultiplier is used as detector, and an Allen[49]-type hydrogen discharge tube as a source of continuous radiation. The available light intensity[10] is such that, with a bandwidth of 17Å, the smallest object area that can be measured is about $5\mu^2$ if the optical extinction is not greater than 1·0. The Land system employs the principle first suggested by BRUMBERG[50] whereby photomicrographs taken at three different ultraviolet wavelengths are converted into visible colours. When these are projected into one image the variation in hue from point to point in the image is some function of the ultraviolet absorption, which bears some relationship to chemical composition. The particular advantages of this method as a cytochemical tool have yet to be demonstrated.

For recording the absorption spectrum of a small area of a cell the photoelectric method consumes less total time and is more accurate than the photographic one. The latter has the advantage that from rapid exposures, an advantage in studying living and moving cells, a permanent record is provided from which the extinction or total quantity of absorbing material can be calculated at leisure. A direct recording microdensitometer

considerably accelerates these measurements[51, 53]. In any system precise focusing on unstained material is best accomplished by visible phase contrast illumination, and for this purpose an additional optical train with a moveable phase ring above the ultraviolet objective has been described[52, 53].

In general, the accuracy of photographic photometry increases with the measured extinction, while photoelectric photometry is most accurate in an extinction range 0·3 to 0·7. In photoelectric measurements the accuracy obtainable can be improved either by increasing light intensity or detector sensitivity; this is desirable when the optical extinction of the object is small[43]. However, in biological material it is often not possible to choose the best value for the extinction as in macro work, and in any case as the optical extinction of the specimen increases, other sorts of error, discussed later, are encountered in microspectrometry.

Method II

This method (Figure 2c) is a development of the old microspectroscopic ocular technique, whereby a spectral analysis is made of the entire radiation after it has left the microscope, and used for example by KEILIN[54] in a study of the cytochrome system of living cells and their extracts. Its application to the ultraviolet region has been made possible by the development of achromatic u.v. objectives. In the technique first described by JOPE[55] (see also BARER, HOLIDAY and JOPE[56]; SEEDS and WILKINS[57]; MELLORS[58]) and used in an analysis of blood cells the microscope image is formed in the slit of a spectrograph which analyses the total radiation and records it on a photographic plate. Its main advantage is that from a single exposure the complete absorption spectrum can be deduced. However, when a mercury arc is used as source, owing to the great variation in line intensity, several different exposures are required to obtain the appropriate photographic densities and this increases the risk of photolytic damage. This possibility of damage makes it undesirable to scan the spectral images photoelectrically. When the image intensity varies across the width of slit used, and with a continuous source (hydrogen or Xenon arc), then obviously errors will arise in amount depending both on the extinction of the object and the variation in it. Similar effects[57] will occur even with a line spectrum such as that from a mercury arc where some of the lines are diffuse or multiple and some continuum is present.

ABSORPTION IN THE OBJECT AND IMAGE FORMATION

CASPERSSON[8] (1936) in a fundamental paper has given a theoretical discussion of the possibilities and limitations of absorption measurements with the microscope. The following results show how data on the extinction

of the object may be obtained from measurements of the light intensity in the *plane* of *an* image. It has been found convenient (see also CASPERSSON[9], 1950) to consider (1) homogeneous objects large in size compared with the wavelength of the light used; (2) the lower limits of size for which absorption measurements, to a required degree of accuracy, can be made; (3) the measurements of total amount of substances; (4) the effects of an inhomogeneous distribution of absorbing material, and (5) certain additional sources of error.

1. *Homogeneous objects large in size compared with the wavelength of light*

For large objects image formation in the microscope is adequately described, apart from diffraction at the edges of the object, by geometrical optics and calculations have been made of the distribution of light intensity in the image plane of some simple geometric bodies which serve as models for the shapes encountered in biological material. In the simplest case, when a plate of uniform thickness in a plane at right-angles to the microscope axis is illuminated by a uniform beam of parallel light, the intensity distribution in the image plane, apart from a scale factor, is the same as that in a plane immediately behind the object. From energy measurements in the image plane the extinction of the object can be calculated in the usual way. It is desirable, even if only to increase the energy available, to use a finite aperture in the illuminating beam and this results in an increase in the path length of the rays in the plate by a factor equal to the reciprocal of the cosine of their angles of obliquity. However, calculations given in a convenient graphical form by BLOUT *et al.*[24] show that the errors introduced by assuming the path length to be the same as that for rays incident normally are not serious at low condenser apertures.

When a uniform refracting and absorbing sphere is illuminated by parallel light the intensity distribution in the image, formed when the microscope is focused on a plane through the centre of the sphere, is no longer identical with that in a plane immediately behind the object. However, calculations (see also THORELL[46]) for both parallel and convergent light, and for refracting and non-refracting material, show that up to relatively large distances from the centre of this image (of the order of 0·2 to 0·3 times the radius) the light intensity is, with negligible error, the same as that for a plane parallel plate of the same material, and thickness equal to the sphere diameter, illuminated normally with parallel light. These considerations can be extended to include other models such as cylinders and ellipsoids.

Errors arise in any absorption measurements when the decrease in light intensity is not caused by absorption alone, as may occur in these models by refraction or reflection. Reflection losses, for example, occur at interfaces where the refractive index changes abruptly and can be calculated

by FRESNEL's equations (HARDY and PERRIN[40], p.27). However, even for the extremely high refractive index of 1·5 (dry protein film in air) the light losses at normal incidence are only 8 per cent ($E \sim 0{\cdot}037$) and this value is unlikely to occur in cells immersed in liquid media. For small particles the loss of light is due to diffraction or scattering, and FRESNEL's equations can no longer be applied.

2. *Object size of the order of the wavelength of light*

The extent to which absorption measurements can be made on objects which approach in size the wavelength of light is an important practical question, since many cell structures and certain unicellular organisms are of these dimensions. CASPERSSON[8] has estimated this lower limit of size in two ways. One method was to determine the smallest object around which the light intensity distribution could be calculated according to the simple ray optics used above. The distribution around a spherical particle of any size is given by the MIE[62] theory, in terms of the particle diameter, the wavelength λ of the incident light, and the relative complex refractive index n'

$$n' = n\,(1 - i\gamma) \qquad \ldots . (3)$$

$$\gamma = \frac{a\lambda}{4\pi} \qquad \ldots . (4)$$

$$I_T = I_o\, e^{-ad} \qquad \ldots . (5)$$

where n is the ordinary relative refractive index, i equals $\sqrt{-1}$, and γ the absorption index is related in the manner shown (4) to the absorption coefficient a in the usual absorption equation (5). In the extreme case for particles with a diameter of less than about one-tenth the wavelength the light is diffracted all around them as described by the RAYLEIGH[63] theory. Calculations[59, 60, 61, 8] for non-absorbing particles ($\gamma = 0$) using the MIE theory showed that the light distribution around a spherical particle agreed with that calculated from geometrical optics for a diameter of about three times the wavelength of the light used, that is about 0·8 μ at 265 mμ. As the size of the sphere decreases, an increasing and not easily calculable proportion of the light energy is diffracted outside the angular cone which can be collected by the highest aperture objectives.

The second method was to determine the size limit below which the image formed by the objective, assumed to be perfect, was no longer a true representation of the object. This means that at the lower size limit, the distribution of light intensity in the image of the two dimensional objects considered in the analysis is similar to a required degree

of accuracy, to that in a plane immediately behind the object. For this purpose the ABBE[64] diffraction theory of microscope image formation was used, according to which a complete representation of the object requires the cooperation of all the diffraction spectra from it. The calculations showed that the diffracted light from a slit aperture (the same by BABINET'S principle as from a strip object) illuminated by a coherent beam of parallel light is collected, with an energy loss of only 1 or 2 per cent, by an objective of 1·3 N.A. for a slit width of 0·5 to 0·75 μ at a wavelength of 275 mμ, a dimension similar to that obtained by the first method. CASPERSSON concluded that objects should be greater in size than three to four times the wavelength of the light used if accurate absorption measurements are to be made. In practice biologists frequently wish to measure objects smaller than this.

WILKINS[65] has recently applied RAYLEIGH[66] microscope theory to calculate the light distribution in the image of strip objects. Each point in the object field is regarded as self-luminous, corresponding more closely to the conditions prevailing when a finite condenser aperture is used. Each point then gives rise to the well-known Airy disc intensity distribution in the image, the overlapping of the diffraction patterns from all points in the object resulting in the image. This treatment shows clearly how light is diffracted into the image of narrow opaque objects, producing a spurious transmission. It further shows that even for objects of width equal to the wavelength the percentage errors which decrease with decreasing optical extinction approach limits which may be tolerated. However, the nature of the theory precludes consideration of light energy diffracted outside the objective, and for this reason may be inadequate. Additional factors complicate absorption measurements on small objects. For example, it is well-known that small, perfectly transparent but refractile bodies show dark and light contours under the microscope, reversing in appearance through focus, and most apparent at relatively low condenser apertures. These phase effects[65] are demonstrated in Figure 3A; at *a* the mitochondrium appears as a non-absorbing filament with a dark halo, and at *b* as an absorbing filament with a light halo. At a slightly different focus the effect is reversed. Clearly, unless a correct focus, that is the position at which for a non-absorbing wavelength there is no intensity variation, can be chosen and maintained at other wavelengths valid absorption measurements on single small particles cannot be made.

3. *Total amounts of absorbing material*

It follows from LAMBERT and BEER'S law that if E is the optical extinction of a uniform slab of absorbing material of area A cm^2, then the total mass M contained in it is given by

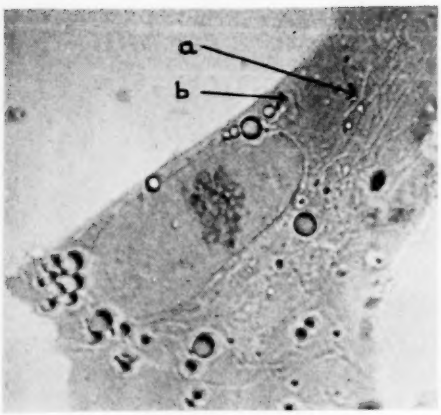
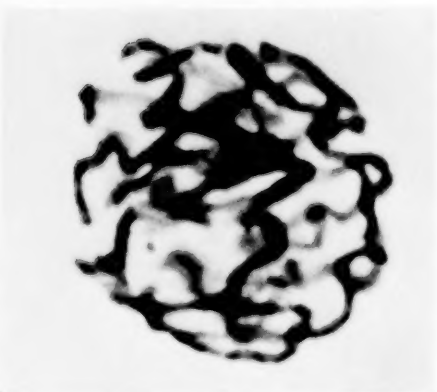


FIGURE 3. A. Living chick heart fibroblast in tissue culture, λ 265 mμ. Objective N.A., 1.24. Condenser N.A. 0.6.
(Taken from DAVIES[67])

B. Chick heart fibroblast, prophase. Formalin fixation, Feulgen stained.



C. Onion root tip. Prophase. Carnoy fixed. Feulgen stained.

D. Onion root tip. Metaphase. Carnoy fixed, Feulgen stained.

B, C and D were taken at λ 546 mμ. Objective N.A. 1.32. Condenser N.A. 0.6. Magn. $\times$2300.

$$M = \frac{E.A}{k} \text{ mg.} \qquad \ldots . (6)$$

where k is a constant for the particular material and is equal in value to the optical extinction of a solution of concentration of 1 mg. per cc in a path length of 1 cm, and may be obtained by separate spectrophotometric measurement. Thus, a value for the total mass is obtained without reference to the thickness which, in biological material, may be very difficult to measure. When the extinction varies, its average value must be obtained from a series of extinction measurements traced across the projected area of the cell structure measured. If a_x denotes the length of a trace, E_x, the extinction, and n is the number of traces taken, then M is given by

$$M = \frac{1}{k} \frac{\sum_{x=1}^{n} a_x E_x}{\sum_{x=1}^{n} a_x} \times \frac{\text{PROJECTED AREA}}{\text{m}^2} \qquad \ldots . (7)$$

where m is the magnification of projection. E_x usually varies along the trace and the product $E_x.a_x$ is obtained by measuring the area under it. This method (see CASPERSSON[9]) has been used by WALKER and DAVIES[53] to measure the total amounts of absorbing material in the 'nuclei' of fibroblasts grown in tissue culture (see Figure 3A). When the projected area is not well-defined or is difficult to measure (see Figure 3B) then a slightly different procedure is required. If a_x and E_x denote the same quantities as before, and if w is the total width of projected structures in a direction at right-angles to the traces, then M is given by

$$M = \frac{1}{k} \frac{\sum_{x=1}^{n} a_x E_x . w}{m_1 \, m_2 \cdot n} \qquad \ldots . (8)$$

where m_1 is the magnification of projection and m_2 is the magnification on to the extinction trace. The required number of traces, n, depends on the degree of heterogeneity.

While the above procedures may also be used for the measurement of the total amount of material in non-refracting spherical or ellipsoidal bodies, another method has been mainly used for the photoelectric measurement of stained nuclei. RIS and MIRSKY[68] have measured the total energy incident on and transmitted by the entire spherical nucleus, and if E_1 is the measured extinction and r the radius then M is given by

$$M = \frac{1}{k} E_1 \cdot \pi r^2 \qquad \ldots . (9)$$

A disadvantage of this method, pointed out by them, is that nuclei are rarely perfectly spherical (or uniformly absorbing) so that the enclosing diaphragm lets through light from clear regions of the field, giving rise to errors as discussed below. These errors are small if the extinction is sufficiently small ($< 0{\cdot}1$). Errors would also arise if the objects were refracting. Another method[68] which obviates errors due to refraction is to measure the extinction E_2 of a central cylindrical plug, when M is given by

$$M = \frac{1}{k} E_2 \cdot \pi r^2 \cdot \frac{1}{F_1} \qquad \ldots . (10)$$

where F_1 is the ratio of the volume of a cylinder of diameter and height equal to the sphere diameter to that of the sphere, and has the value $^3/_2$. SWIFT[69] has measured the extinction E_3 using a limiting diaphragm of radius R which can no longer be regarded as small compared with the sphere radius r, when M is given by

$$M = \frac{1}{k} E_3 \pi R^2 \cdot \frac{1}{F_2} \qquad \ldots . (11)$$

where F_2 is the fraction of the nuclear volume included in the measured cylinder and is given by

$$F_2 = \frac{r^3 - (r^2 - R^2)^{3/2}}{r^3} \qquad \ldots . (12)$$

In the above treatment the rays have been considered as passing parallel through the sphere, and this seems justified since it has been shown[8,46] that the light distribution in the image of a non-refracting sphere (corresponding fairly closely to mounted stained material) is the same within 1 per cent whether parallel or convergent light is used, and also up to quite a large distance from the image centre for spheres of low refractive index. When the bodies are best treated as ellipsoids the product of the major and minor axes has been used in measuring the projected area.

We have already seen that phase effects, diffraction, and to these may be added objective aberrations[65], may alter the energy distribution in the images of small bodies. These are essentially interference phenomena, in which a constant amount of light energy is only redistributed over the image plane. It is useful to consider the relative errors resulting in both extinction measurements and integration measurements of total quantity of absorbing material. An extreme example, with the maximum expected error is that of an absorbing body which goes completely out of focus; the measured extinction approaches zero but the energy absorbed by the body remains constant. The expression[70] for the percentage error ϵ in total amount of material is

$$\epsilon = 100 \cdot \frac{ad - (1 - e^{-ad})}{ad} \sim 50ad\ \% \qquad \ldots . (13)$$

where ad is the exponent in equation (5). Where the initial optical extinction of the object is E the error is given by the expression

$$\epsilon \sim 116\,E\ \% \qquad \ldots . (14)$$

Thus, for objects of extinction, say 0·1, which go completely out of focus so that the extinction becomes zero, the error in total amount is only about 12 per cent. The equations also show how the errors increase with particle extinction.

4. *Inhomogeneous objects*

The above sections have dealt mainly with geometrically well-defined objects in which the distribution of material is a homogeneous one. However, the majority of cells are characteristically inhomogeneous and the particulate nature of the protoplasm is usually enhanced after fixation. The two kinds of error, which arise as a result of this discrete spatial distribution, are major factors in promoting the present uncertainty[71] as to the accuracy of microspectrographic measurements. Non-specific light loss as a result of scattering outside the collecting aperture by particles

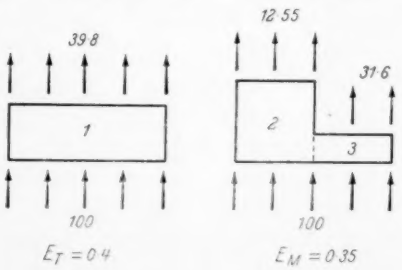


Figure 4. When the transmission varies across the object the extinction no longer accurately measures the amount of material in the beam. When the material in plate 1 of extinction (E_T) 0.4 is *redistributed* into plates 2 and 3 of extinctions 0.6 and 0.2 respectively the measured extinction (E_M) is 0.35.

ranging in size from about the wavelength of light downwards makes the measured optical extinction too high. The measured extinction of a volume in which the material is inhomogeneously distributed in bodies of any size may be less than if the same quantity of material was evenly spread throughout the same volume. These errors arise as a result of variation in transmission in the object (Figure 4). Their magnitude depends on the original nature of the biological material, the methods of preparation and of measurement.

(*a*) *Non-specific light loss*—The extent of the non-specific light loss is indicated by the optical extinction at a wavelength for which there is no real absorption; for a nucleoprotein this is in the region of 320 mμ. Data

in the literature[124] shows that in fixed material this extinction may often be seriously high, as much as 50 per cent. of that at 260 mμ near the absorption maximum. According to an early proposal made by CASPERSSON[72] the magnitude of the light scatter in the presence of real absorption may be calculated from the measured extinction at 320 mμ by extrapolation using RAYLEIGH'S law. This law holds for particle sizes of about one-tenth the wavelength and less, and states that the turbidity (the absorption coefficient due to scattering) varies inversely as the fourth power of the wavelength. For several reasons this procedure cannot be considered entirely satisfactory for quantitative measurements. Many of the particles in cells are of the order of the wavelength of light in size and it has been shown[59,60] (see also OSTER[73]) that for a constant weight concentration of material the extinction due to scatter is a maximum for precisely such particles and is relatively small for those about a tenth the size. For the larger particles turbidity varies inversely as the square of the wavelength (JOBST[74]). No precise value can be given to the wavelength exponent since, apart from the range in particle size occurring, the collecting aperture is not small, as in turbidity measurements, and with change in wavelength the angular distribution of the scattered light varies.

According to the general MIE theory light diffraction is also a function of the real absorption (imaginary part of the complex refractive index, expression (3)). Using RAYLEIGH-GANS[75] theory, STOKES[76] has calculated the light distribution around particles (spheres, and randomly oriented plates and rods) with only real absorption and no refraction. For a collecting numerical aperture of 1·3 the worst possible particle size for scattering is about one-half the wavelength, and even with an absorption corresponding to 100 per cent nucleic acid ($E \sim 0{\cdot}28$), unlikely to occur in practice, the error (expressed as the ratio of the energy scattered and not collected to that absorbed) is small—about 5 per cent. It can, in general, be ignored. Finally, on the long wavelength side of an absorption band, and depending on its magnitude, the real refractive index affecting the light scatter is increasing. There is no experimental data available on the refractive indices of cell proteins in the range 320 to 240 mμ prior to the general increase in protein absorption below this wavelength. As a result of such considerations of this general kind CASPERSSON[9] has more recently proposed that the scatter should be estimated from an experimental measurement of the angular distribution of light around the object under examination. In the absence of the appropriate data on the particle shape, size and especially the relative refractive index it would be impossible to compute from a measurement in visible light, using the MIE theory for spherical particles, the appropriate ultraviolet correction. This method has not yet been used on the ultraviolet, where purely technical difficulties might be experienced, owing to the low light flux available. Non-specific light loss

should in practice be kept at a minimum by suitable fixation (CASPERSSON[43] see p. 218) and by employing a high collecting and relatively low illuminating aperture. Using the optical extinction at 312 mμ, limits to the amount of nucleic acid in the cell may be obtained by assuming that the scatter loss is either independent of wavelength or varies inversely as the fourth power; however, change in refractive index would tend to make the limits too high.

A different approach to the problem of non-specific light losses is that of POLLISTER and RIS[41] who have determined the nucleic acid content of fixed material by measuring the extinction at 265 mμ of the same cell before and after extraction with trichloracetic acid (TCA), which selectively removes nucleic acids (SCHNEIDER[77]). This method assumes that the nucleic acids do not contribute much to the non-specific light loss and that they are completely removed. It is also unlikely that, in general, extraction with T.C.A. will leave the optical properties of the cell unaltered, although it has been found[78] that there is very little change in the scatter in Carnoy fixed chick fibroblasts after removal of the nucleic acids. In quantitative staining methods the unstained cell, whose extinction is usually relatively low, may be used as a blank[41].

(*b*) *Distributional errors*

(*i*) *Quasi two-dimensional objects*—Consider as an example the Feulgen stained prophase nucleus of a chick fibroblast (Figure 3B) where all the chromosomes lie in much the same plane except that they have clear gaps between them. When the total amount of absorbing material in them is determined, by measuring the total energy incident on and transmitted by the entire nuclear area, there is an error which is a function of the extinction per chromosome and the fraction of the total area occupied by them. GLICK *et al.*[79] have recently given a two-dimensional treatment of the errors, applicable to the above case, showing how the errors increase with particle extinction. There is an accurate way of determining the total amount of material in the nucleus and that is by scanning with a small aperture over an image in which the chromosomes are resolved. However, as was pointed out on page 208 (see also formula (14)), errors may still occur from any cause tending to blur the individual detail, such as some particles not being within the focus of the objective or, even when this is the case, as a result of the diffraction blurring of the smaller particles.

(*ii*) *Three-dimensional objects*—Consider a thick plate in which the material, initially homogeneously distributed, is concentrated into small, similar discs randomly distributed and filling the same space as before; it is assumed that the discs are at right-angles to the axis of the microscope

and to the parallel illuminating beam, and that ray optics can be used *i.e.* no diffraction takes place. In a plane behind the object the light intensity fluctuates since each ray strikes a different number of particles. It can be shown[76] that, as a result of this fluctuation, the percentage error in determining the total amount of material by measuring the total energy incident on and transmitted by the entire area is $\sim 116\,E$, where E is the extinction per disc (this result is the same as that given by expression (14)). The correct value for the total amount could be obtained by integrating over the image plane. With parallel light the depth of focus is infinite so that all particles are in focus, and any image plane will serve for the measurement since the intensity distribution in all planes is similar apart from a scale factor. For convergent light, and refracting or diffracting particles, only a fraction of the total number can be focused, and the gain in accuracy by integrating over a focused image (rather than measuring the average light intensity in the image plane) will depend on the value of this fraction and will be significant only when the fraction is large; when the distribution is not random the gain may be greater. It was pointed out that calculations have been made of the relationship between the absorption and the light intensity distribution in the image of certain simple homogeneous model shapes. When the microscope is focused on a plane through the centre of the roughly spherical, Feulgen stained, onion prophase nucleus (Figure 3C) errors will arise as a result of the large degree of inhomogeneity. The onion metaphase plate (Figure 3D) is of an irregular shape, not even approximating to one of the simple models, and it is also unsuitable for accurate microspectrographic measurements.

We can use the above treatment, together with auxiliary data on the extinction per particle, to obtain a rough estimate of the order of magnitude of the error which may be encountered. For particles of about 1 μ in size or greater the optical extinction may be determined approximately by experiment; for smaller particles it is necessary to use such data as may exist on the chemical composition of the cell material and the stoichiometry of any staining reaction employed. For example, when the desoxyribose nucleic acid content of nuclei is measured by its ultraviolet absorption the biochemical extraction data of ALLFREY, STERN, MIRSKY and SAETREN[80] may be used; it shows that the protein/D.N.A. ratio varies between three and six. This means that 1 μ thick particles of these compositions will have optical extinctions of 0·5 and 0·28 respectively, and the error in measuring a random three-dimensional distribution, according to the simple treatment given above, is about 60 per cent and 30 per cent; for 0·1μ particles the errors will be 6 per cent and 3 per cent. In living cells where the particles may also contain water the errors are smaller. Rough estimates of error can be made in this way for the particular cells investigated.

5. *Additional sources of error*

(*a*) *Glare*—As a result of glare[81,82] (that is, light reflection and scattering by components such as objective-surfaces between the specimen and the detector), light from an object point no longer proceeds to the conjugate image point but is spread over the image field, and extinctions will appear lower than they really are. The magnitude of the effect increases with increasing condenser aperture and field size, and therefore these should be reduced to the minimum size compatible with other requirements. Glare may be estimated from the measured extinction of an opaque object[65] of such a size ($> 1\ \mu$) that the amount of light diffracted into it is small; the object is preferably located in a field where the material is distributed in a manner similar to that encountered in the actual measurement.

Consider an object of area a, located in a field of area $(A + a)$. In the absence of glare the true extinction E_T is given by

$$E_T = \log_{10} \frac{I_o}{I_T} \qquad \ldots . (15a)$$

where I_o and I_T are the incident and transmitted intensities respectively. In the presence of glare the apparent extinction E_A is given by

$$E_M = \log_{10} \frac{I_o + \kappa (I_o A + I_T a)}{I_T + \kappa (I_o A + I_T a)} \qquad \ldots . (15b)$$

where it is assumed that a fraction of the energy through each point in the field is scattered over the image plane. The constant κ is related to the measured extinction E_M^{∞} of an opaque body of area a by the equation :

$$E_M^{\infty} = \log_{10} \frac{1 + \kappa A}{\kappa A} \qquad \ldots . (15c)$$

It will be seen (Figure 5) that errors due to glare increase with the optical extinction of the object.

Glare is likely to be less in simple reflecting objectives than in multi-lens refractors. An effect, comparable to glare, whereby light is diffracted into the images of fairly large objects arises in reflecting objectives where hand-figuring is used, producing cobbled surfaces (WILKINS[65]).

(*b*) *Molecular orientation*—Errors in absorption measurements arising as a result of molecular orientation have recently been considered by COMMONER and LIPKIN[83,84], particularly in the estimation of nucleic acids from their absorption at about 260 mμ. If in any structure optically anisotropic molecules are oriented so that absorption depends on the direction of the electric vector in the incident beam, dichroism will result and measurements made with unpolarised light will give a value for the number of absorbing molecules different from what it would be if they

were unoriented. Dichroism has been found by CASPERSSON[86] (see also SEEDS, this volume) in partially oriented films of thymus nucleic acid and in the exceptional case of certain sperm heads. However, according to THORELL and RUCH[87], in other cell structures where orientation might be expected (for example in salivary gland chromosomes) the measured ultraviolet dichroism is low and in general the error introduced in absorption using non-polarised light is negligible. Similar conclusions were reached by WILKINS[65] and POLLISTER and SWIFT[85].

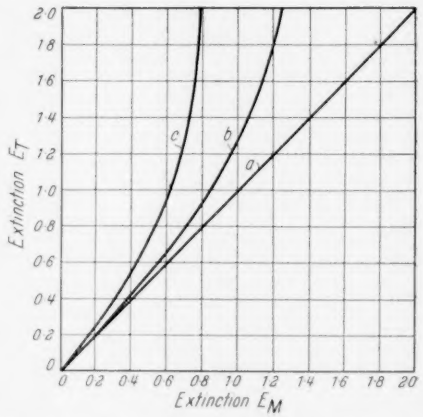


Figure 5. Shows the relationship between true extinction E_T and apparent extinction E_A for varying amounts of glare, corresponding to values of E_M^{∞} (the apparent extinction of an opaque object) $\infty(a)$, 1.24(b) and 0.8(c). $a \ll A$. The corresponding ratios of light intensity in the image of the opaque object to that in the rest of the field are 0, 4.75 per cent and 15.8 per cent.

Data from the above sections can be used to choose the best values of objective and condenser aperture for microspectrometry. When the object scatters or diffracts the incident light, then the objective should have a high aperture. For large three-dimensional objects, homogeneous or otherwise, the best condenser aperture is a relatively low one[9]; as a rough guide say $\frac{1}{2}$ to $\frac{1}{3}$ that of the objective. Errors due to variation in path length (through plate-like objects), non-specific light loss, inhomogeneity and glare are then reduced. However, the light flux through the microscope decreases with condenser aperture. For small objects, lowering the condenser aperture distorts the light intensity distribution in the image.

BEER'S LAW, INTRA- AND EXTRA-CELLULAR ABSORPTION

It is well known that BEER's law is not universally valid for absorbing molecules and that changes in concentration may give rise to a non-proportional changes in extinction as a result of interaction either among the molecules of solute themselves or with the solvent. However, it has been shown to hold for some important biological molecules in solution *in vitro*, for oxyhaemoglobin in the concentration range 0·019 per cent to 38·8 per cent, JOPE[55] (see also THORELL[88]), and for purified ribose and

desoxyribose nucleic acids in the concentration range 0·005 per cent to 2·5 per cent (THORELL[46]). In these experiments an upper limit is set to the concentration range owing to the difficulty of measuring cuvette thickness; the range covered is usually not sufficient to include the higher concentrations encountered in the cell particles in some fixed material. The results obtained for these particular biological molecules are in marked contrast to the behaviour of some dyes studied by SHEPPARD[89] where, as a result of molecular interaction, marked deviations from BEER's law arise.

In the quantitative interpretation of the absorption spectra of cell structures it is usually assumed that each substance exhibits the same spectral absorption in the cell as it does in solution outside the cell. This assumption may not always be valid, since, in cells, substances are in a different environment (such as hydrogen ion and salt concentration) and may be combined with other components or differ in their degree of polymerisation; apart from effects due to changes in concentration, each of these factors may affect the spectral absorption in varying degree. Changes in the absorption spectrum of a molecule are known to arise as a result of linkage with other molecules; for example a band shift of about 10 to 35 Å occurs in the spectrum of the aromatic amino acids when they are incorporated into a protein structure (discussed by BEAVAN *et al.*[90]). The spectral bands due to the haem in blood cells depends to a slight extent on the nature of the protein carrier (JOPE[55]); the degradation of highly polymerised ribonucleic acid, with alkali, to lower nucleotides results in a 30 per cent. increase in absorption and a similar increase occurs when desoxyribose nucleic acid is degraded with desoxyribonuclease (KUNITZ[91], MAGASANIK and CHARGAFF[92]). Preliminary experiments reported by BRACHET[93] also appear to indicate that some proteins and nucleic acid in combination show a spectral absorption not obtained by the simple arithmetic addition of the separate curves. This happens when lysozyme (but not histone) is combined with sodium thymonucleate. Of course, positional changes in absorption maxima may also occur purely as a result of curve addition.

Changes in absorption between cell and solution have been demonstrated by COMMONER[94]; in Coleus leaf cells the intra- and extra-cellular absorption maxima of the flavone and anthocyanins differ in position. Since the absorption spectra of these compounds vary with hydrogen ion concentration this might be due to a difference in *p*H between cell and solution; however, combination with cell proteins was considered as a more likely explanation. According to MIRSKY and POLLISTER[95] purified histones have an absorption maximum at the usual protein position, 280 mμ, but CASPERSSON[96,97] finds that histones together with nucleic acids in cells have their absorption maximum shifted to 290 mμ. JOPE[55] showed

that while the band systems of tryptophane and phenyl alanine in both adult and foetal haemoglobin are the same in intracellular protein as in solution, an extra band in the longer ultraviolet detected in the cell was not present in solutions. While shifts in band position can be recorded, especially using a continuous spectrum, changes in extinction coefficient between cell and solution would be difficult to demonstrate. Present indications are that these secondary phenomena do not greatly interfere with the ultraviolet microspectrographic assay of desoxyribose nucleic acids in some living and fixed differentiated nuclei. KURNICK and THORELL[126,127], WALKER and YATES[98,99] and LEUCHTENBERGER, LEUCHTENBERGER, VENDRELY and VENDRELY[100] have found fairly good agreement between micro-assay of D.N.A. in individual nuclei, using extinction values from dilute solutions of purified D.N.A., with the values given by bulk biochemical methods.

While on the one hand the effects discussed above may be considered as interfering with quantitative cell absorption measurements, it is more profitable to view any differences in spectral absorption of substances between the living or fixed cell and solutions as possibly providing extra information about the special conditions existing in the cell itself.

ULTRAVIOLET MICROSPECTROGRAPHY OF LIVING CELLS

Ultraviolet microspectrography of living cells would appear to offer the important possibility of following the changes in amounts of absorbing material in an individual cell during its life cycle. However, as is well-known, ultraviolet light damages cells, and whether this will be a limiting factor can be determined by comparing the dosage required for micro-absorption measurements with the dosage which is known from previous work to injure the cell. The latter subject has been extensively reviewed[101–104].

The factors [105,106] affecting the dosage to the cells using photographic recording are :

(i) Properties of photographic emulsions,
(ii) Optical extinction of the cell and light losses in stages after the cell,
(iii) The precision of recorded data.

Serial photomicrographs of living cells are conveniently made on 35 mm film and the optimum combination of film speed and resolving power, can be selected by using the experimental data of FRASER[107]. Some of the best films available at present are *Kodak Microfile Panchromatic, Kodak Recording Film* 1372, and *Kodak Spectrum Analysis No.* 1. The light transmission of some ultraviolet objectives has been measured by LOOFBOUROW[11] and DAVIES[105] who found a marked reduction at shorter wavelengths. Freshly aluminised reflecting objectives without added refracting components

gave the highest transmission, but this decreased with time owing to deterioration of the surfaces. The magnification on the film must be just sufficient for recording the finest resolved detail. Any greater magnification will involve an unnecessary irradiation of the cell, and with less magnification detail will be lost. The choice may be made experimentally by varying the magnification on to the film and noting, on suitable enlargements, when the smallest structure is just resolved by the film. When the best photographic material and objective have been chosen, the minimum dose will depend on the microscopically resolved detail (greatest for high aperture objectives) which is required in the photomicrograph.

The dosage required for photomicrography can be determined either from the dose-rate measured by a calibrated thermopile located in the plane of the object or, alternatively, calculated from the measured film sensitivity from the relationship

$$E_\lambda = m^2 \cdot F_\lambda^d \, T_\lambda \qquad \ldots . (16)$$

where E_λ is the dosage to the cells in ergs. cm^{-2} at a wavelength λ, m the magnification on to the film; F_λ^d is the film sensitivity in ergs. cm^{-2} for a density d, and T_λ is the objective transmission. DAVIES[105] has found that under optimum conditions the radiation dose required for photomicrography of cells in tissue culture (chick fibroblasts) with a reflecting objective of 1·3 N.A. is about $0{\cdot}5 \times 10^4$ ergs. cm^{-2}. These measurements are comparable with those of MELLORS[106] *et al.* when the relative amounts of information obtained are taken into account. This dosage has been compared with the extensive results of MAYER and SCHREIBER[108] on the effects of measured quantities of ultraviolet radiation on cultures of chick fibroblasts. It was concluded that, under optimum conditions, only one or two photographs of chick fibroblasts can be taken at 265 mμ, and that a complete absorption spectrum cannot be recorded without damage to the cell. However, the effects of the ultraviolet (changes in absorption and morphological changes) do not appear until some time after exposure, so that while the changes in absorption in a cell cannot be recorded serially, valid absorption spectra can be obtained by working within this restricted time interval. Data on some other biological materials can similarly be deduced by reference to the literature.

Calculations have been made (DAVIES[105], p. 397) to find whether any reduction in dosage may be expected when cell extinctions are recorded photoelectrically. It is then convenient to measure the absorption of a small area of the cell without irradiating the rest of it. To obtain data on the whole of an irregularly absorbing cell, as is done photographically, it would be necessary to use some sort of scanning technique. A way of making a proper comparison is to find the relative amounts of energy required

photographically and photoelectrically to obtain the optical extinction of the *same* area of the cell to the same degree of accuracy. This accuracy is that of recording cell extinction and is to be clearly distinguished from other errors; for example, those arising from the peculiar nature of the biological material.

When all purely calibration errors have been eliminated from the photographic method errors still arise due to uneven distribution of the silver grains and, for a given magnification on to the film, the resulting random error or noise produced in the microphotometer trace sets a limit to the size of object which may be measured to a required degree of accuracy. For example, when *Kodak Microfile Pan*, a fine grain emulsion is used, at × 100 magnification, the smallest object of apparent optical extinction 0·3 that can be measured to an accuracy of ± 2 per cent has an area of about $0{\cdot}2\mu^2$; this is larger than that element which can just be resolved by the film. For quantitative studies, recording the noise due to graininess provides the best method of choosing the proper magnification on the film.

Photoelectrically, a limit is set to the lowest light flux measurable to a required degree of accuracy, by the quantum efficiency of the photo-cathode and the statistical fluctuations in the photoelectrons and accompanying thermionic emission from the photo-cathode. The R.C.A. I.P. 28 photomultiplier with a quantum efficiency of 7 per cent, and in a circuit with a response time of $\frac{1}{4}$ second, was compared with the best film. It was found that the energy required to measure the same area to the same degree of accuracy (± 2 per cent) was about 1/7 of that required photographically. By eliminating thermal noise, which can be done by cooling the photo-cathode[193], the above fraction is decreased to about 1/30. These calculations indicate that it should be possible, with suitable photoelectric recording, to follow the changes in total absorption of some living cells during growth without damaging them.

FIXATION

Absorption measurements on living cells are limited to particular classes of chemical substances and restricted to certain cell types which can be individually observed in the microscope. An *important limitation* of microspectrographic measurements is that over- and underlying material will often contribute to the absorption of that particular volume whose absorption is required. For example, the nucleolus (Figure 3A) has, above and below it, nuclear and cytoplasmic material whose precise absorption would be difficult to ascertain. COMMONER[109] has used some special cells which have a well-defined and measurable geometry. It is sometimes possible, with fixed material, to overcome these difficulties by sectioning.

The changes induced in cells as a result of fixation raise additional problems for quantitative microspectrometry and they may be discussed under the general headings: I. Removal and redistribution of material; II. Changes in the optical and chemical properties of the remaining material. It is now known that, with the usual chemical fixatives, large quantities of cellular material may be lost. SYLVÉN[110,111], for example, has found this to be the case by an analysis of the remaining fluids after fixation of fresh biological material in bulk, and ENGSTRÖM[112] (see also NURNBERGER, ENGSTRÖM and LINDSTRÖM[113]) using ultraviolet microscopy and mass analysis by X-rays to compare the effects of various fixatives on cat spinal cord, showed that losses of substance and changes in distribution occurred during routine histological procedures. DAVIES[114] has found changes which may be ascribed to a loss of absorbing material, when he compared the optical properties and integrated absorption in the same chick fibroblast, living and after chemical fixation. To quote one extreme case, the integrated cytoplasmic absorption of living fibroblasts was reduced by about forty per cent after standard formalin fixation. Freeze drying, according to ALTMANN and GERSH, is generally regarded as a procedure during which there is little likelihood of loss or redistribution of material; this technique has recently been reviewed by BELL[115]. Neither freezing nor dehydration renders tissue proteins insoluble so that, prior to cytochemical analysis in aqueous media, cellular substances must be fixed and changes may then occur. Even immersion of the frozen dried sample in doubly distilled glycerol, the method often used for ultraviolet optical analysis, may sometimes cause loss of material and this requires further investigation.

It has been found that the non-specific light loss in some living cells is negligibly small, especially in those areas which appear homogeneous in the microscope. This is not always the case, however, and when there is a particulate distribution of material, in size of the order of the wavelength and of different refractive index from the surroundings, as for example in the living granulocytes studied by THORELL[46], non-specific light losses increase especially in the ultraviolet. Visible dark ground microscopy gives a good indication of the regions where loss due to scatter may be expected. Non-specific light losses generally increase after fixation as a result of the particulate distribution of protoplasm produced, the particles often differing markedly in refractive index from the embedding medium and by a maximum amount for those fixatives acting as protein precipitants. The commonly used embedding media for the ultraviolet fall short of the ideal requirement that they should match both the refractive index and dispersion of the cellular proteins. According to comparative measurements made by CASPERSSON[43] on frozen dried thyroid follicle immersed in either paraffin or double distilled glycerol, despite the higher refractive index

of the former, the latter medium results in considerably less light scatter. This has been attributed to a change from the sudden jump in refractive index at the boundary between protein and embedding medium, to a continuous transition in the value of the refractive index from one phase to the other. Other errors arising from an inhomogeneous distribution have already been discussed. Wherever possible a fixative which does not introduce extra inhomogeneity should be chosen.

Especially with chemical fixation, complex reactions occur with cell substances so that new chemical groups may be formed and others eliminated; HERRIOT[116] has reviewed the numerous reactions between cell proteins and formalin. It is not surprising, therefore, that colour reactions vary in intensity depending on the type of fixation; for example, after formalin fixation the intensity in a properly controlled Feulgen reaction is about double that after Carnoy fixation[69].

While the ultraviolet absorption curves of cells appear to be roughly similar in both living and fixed material, certain detailed spectral changes have been shown to occur after fixation. Large ultraviolet absorption changes can occur as a result of fixation with osmium tetroxide which itself absorbs; a similar increase in absorption was found in model experiments using protein and fat, but not D.N.A. films[114]. Another source of error in microspectrography of fixed material arises from shrinkage or expansion which may often be large, and since the magnitude of the effect varies between cells treated with the same fixative, even relative measurements of concentration (but not of total amounts) may be misleading.

DIFFERENTIAL ABSORPTION METHODS

When cells contain a mixture of absorbing compounds whose spectral bands either overlap or, as is the case for ribose and desoxyribose nucleic acids, are identical, it may be possible to determine quantitatively the components either by curve analysis, the method used by CASPERSSON[72] for nucleic acid protein mixtures, or, alternatively, by differentially removing one component or changing its chemical constitution (by altering those external conditions to which it alone is sensitive) so that its absorption no longer interferes. The method of curve analysis used by CASPERSSON for measuring protein-nucleic acid ratios requires extinction measurements at two wavelengths, namely 260 mμ and 280 mμ. From them the required ratio can be calculated provided the cyclic amino acid content of the protein is known; it has been usual to assume a 'standard protein' containing 5 per cent tyrosine and 1 per cent tryptophane (obtained by using the protein analyses of BLOCK[189]). In practice this method is subject to errors arising both from variation in amino acid content of proteins and difficulty in estimating non-specific light loss. Also the assumption is made

that the absorption of nucleic acid and protein in combination can be obtained directly from the separate absorption curves of the components.

Enzymatic digestion appears to be the most promising method for specifically removing a chosen substance. CASPERSSON[8] (see also CASPERSSON, HAMMARSTEN and HAMMARSTEN[188]) for example, using the ultraviolet microscope, has studied qualitatively the distribution in chromosomes of nucleic acids left behind as the insoluble lanthanum salt after digestion with a protease. The ribonuclease test first described by BRACHET[117] and used in conjunction with basic dyes, has also been employed, again only in a qualitative way, with ultraviolet microscopy[118]. The optimum conditions for this test, including type of fixation, *p*H, buffer, and other variables, have been examined by STOWELL and ZORZOLI[119]. Enzymatic degradation of ribonucleic acid in vitro is known to leave behind an undigested non-dialysable residue, so that for this reason any quantitative results obtained on cells may represent a lower limit to the quantity of ribonucleic acid contained in them. The future quantitative use of enzymes appears highly important and will depend on an examination of the extent to which the substrate is removed completely and specifically, and also the possibility of the alteration in absorption of the remaining component.

According to a differential extraction procedure described by ERICKSON, SAX and OGUR[120] ribose nucleic acid may be removed from fixed tissue by the prolonged action of cold perchloric acid. DAVIES[78] has found, however, that the absorption of fixed chick fibroblasts treated in a similar way increases considerably at wavelengths 312 and 265 mμ. It is not yet clear whether this is due to marked changes in the light scattering or an alteration in the absorption of the remaining desoxyribose nucleic acid, or a combination of both effects.

Single cellular absorption spectra alone cannot distinguish between variously polymerised nucleic acids and nucleotides, and other substances containing purine and pyrimidine rings, nor indeed between free amino acids and those incorporated in proteins, and this is a weakness in present analyses. The detection and especially the interpretation of any small shifts in absorption maxima would be difficult. However, according to HAMMARSTEN[121] biochemical assay shows that mononucleotides are found only in comparatively low concentrations in ordinary cell material; one exception, studied by CASPERSSON and THORELL[122], are muscle fibres containing adenylic acids. In a photometric analysis of extracts of bean root CHAYEN[123] has found, as well as nucleic acids, substances similar in type to ascorbic acid (at neutral *p*H) and catechol with absorption maxima at about 260 mμ and 270 mμ respectively, and both in sufficient amounts per cell to be found by microspectrographic analysis. The ascorbic acid in the extract was determined from absorption measurements before and

after its conversion to the non-absorbing dehydro-ascorbic acid form which takes place on oxidation in the presence of copper ions. A similar procedure appears to hold some promise as a cytochemical method. These results emphasise the need for care in interpreting the ultra-violet absorption spectra of cells, especially in plant material. In microspectrography of Coleus leaf hair cells COMMONER[94] has confirmed the identity of anthocyanins and flavones by noting the similarity between spectral absorption shifts, dependent on *p*H, in cells and in experiments on these substances in vitro. Differential procedures, some examples of which are outlined above, are useful in distinguishing the several components in mixtures of absorbing compounds.

COLOUR REACTIONS

The photometric measurement of colour intensity in cellular structures is subject to the same conditions as those discussed above in measuring naturally occurring absorption. But the accuracy of the method as a measure of chemical constituents is also dependent on, firstly, the dye molecules attaching themselves specifically to the substance measured and, secondly, on there being a stoichiometric relation between the dye molecules and the substance. This latter condition can necessarily only be determined by *in vitro* experiments; it is therefore difficult to demonstrate that these conditions have been rigidly satisfied in the cell. Part of the justification of the technique will have to depend on the consistency of the results, both among themselves and with other complementary techniques. There is therefore the danger of building a complex edifice of theory based on initial false assumptions.

1. *Feulgen staining*

The mechanism of Feulgen staining has been studied quantitatively by several groups of workers[128–130]. DI STEFANO[129] concluded that the optimum hydrolysis time corresponding to the greatest development of stain intensity is reached when the purine part of the D.N.A. molecule has been removed, unmasking the reactive aldehyde groups which restores the colour to Schiffs' reagent. Further hydrolysis removes the pyrimidine part of the molecule and the intensity of the stain decreases. It has also been shown that different fixatives influence not only the rate of decrease in staining during post-optimal hydrolysis, but also the maximum stain developed in the nucleus.

DI STEFANO[129] considered that the amount of Feulgen staining developed with optimal hydrolysis could be used as an absolute measure of D.N.A. However, the values so calculated for various species of animals are very much less than those found by bulk biochemical methods[68]. ELY and Ross[130] were more cautious. They considered that their results support

the view that the Feulgen reagent is specific for D.N.A., but believe that exact quantitative work would be difficult owing to the many factors influencing the amount of colour developed. Their data does not confirm or deny the contention of STEDMAN and STEDMAN[131] that the dye produced diffuses and is adsorbed on to the 'chromosomin' of the chromosomes and does not, therefore, show the true location of the D.N.A. Other evidence, such as that obtained from ultraviolet photomicrographs of living and fixed cells[1,7], which show absorbing chromosomes excludes the STEDMANS' conclusions.

Despite objections to the validity of the Feulgen reaction as a quantitative cytochemical technique, the results so far obtained show a self-consistency and relative agreement with those obtained by other methods that has for many workers justified its extensive use. It is, however, now generally recognised that the results are only relative and must be expressed in arbitrary units[68]. Only cells receiving identical treatment, preferably on the same slide, may be legitimately compared, although the addition of standard cells[68,132] as controls may to some extent avoid the drawback of having to use arbitrary units.

Quantitative cytochemical results obtained mainly by Feulgen staining, have been recently reviewed by POLLISTER, HIMES and ORNSTEIN[133], VENDRELY[134] and HUGHES[144]. It has been shown that the ratios of amounts of stain in tissues of different animals correspond to the ratios between the D.N.A. values obtained by bulk biochemical extraction. Where a higher average biochemical result was obtained (*e.g.* in rat liver[135]) this has been explained by the presence of cells in classes whose average D.N.A. content is 1, 2 and 4 times the normal diploid value, and this has been shown in certain tissues to correspond to the actual degree of polyploidy[136].

SWIFT[69] has shown that in mouse nuclei ranging in volume from 24 μ^3 to 460 μ^3 the amount of D.N.A. as shown by Feulgen staining is constant. It has been calculated[133] that this represents a range of D.N.A. concentration from 23 per cent to 1·2 per cent. It is interesting to contrast this result obtained from biological objects with the early *in vitro* experiments of CASPERSSON[137], who found that the amount of Feulgen staining was only proportional to the D.N.A. content over a concentration range of 0·02 per cent to 1·5 per cent and then only for purified D.N.A. without protein. It would appear from SWIFT's results, and also those of ALFERT[135] on mouse oocytes, that Feulgen staining is a measure of some constant component of nuclei and that the photometric measurements of Feulgen staining are reasonably accurate over a wide range of concentration and thickness.

It should be noted that the Feulgen extinctions of many animal nuclei are low (0—0·5) and that errors due to inhomogeneity and glare in the instrument are consequently much reduced. Work on certain plant cells[139,140]

has shown a higher range of mean extinctions (0—1·3) where the above errors may be more serious, particularly as these nuclei are often very inhomogeneous. However, BRYAN'S[140] work on Tradescantia meiosis has given results in fair agreement with the biochemical findings of OGUR *et al.*[141] on similar material.

Other quantitative staining methods have been employed or suggested in the study of both nucleic acids and other cellular components, but none of them has been so widely used as the Feulgen reaction. It is consequently less easy to compare the results obtained from these methods with those from other techniques.

2. *Methyl green*

KURNICK and his co-workers[142,143,127] have shown that methyl green combines specifically and stoichiometrically with polymerised D.N.A. in the absence of histone in the proportion of one molecule of dye to 10 atoms of phosphorus. He has shown[127] that calf thymus and mouse liver nuclei contain an amount of polymerised D.N.A. which compares with the values obtained by other methods, although his values for chick erythrocyte nuclei are rather low. LEUCHTENBERGER[145] and POLLISTER and LEUCHTENBERGER[146] have also made measurements of methyl green staining. Their results have shown that during pycnosis or after treatment with hot water, stainability with methyl green, but not with Feulgen, is lost, tending to confirm the specifity of the former stain for polymerised D.N.A. Certain details of their method have, however, been criticised by KURNICK[127].

3. *Millon*

The well-known macro method of detecting tyrosine and tryptophane in proteins by the Millon-mercurial test, used quantitatively by BRAND and KASSEL[190], has been adapted as a cytochemical technique for estimating protein[41]. A standard protein with a given percentage of tyrosine and tryptophane must be assumed, although the proportion of these components may in fact vary considerably. It is interesting to note[191] that the ratio of protein to nucleic acid as measured by the ultraviolet absorption and by the Millon and Feulgen reagents, is higher than that found recently by ALLFREY, STERN, MIRSKY and SAETREN[80] by their method of isolating nuclei in non-aqueous media. However, the published figures of POLLISTER and LEUCHTENBERGER[191] are based on a value for D.N.A. obtained from Feulgen staining according to DI STEFANO'S[129] calculation. ALLFREY *et al.*[80] have also commented on the discrepancy between the proportion of histone to non-histone protein that they found, and the lower proportion found by POLLISTER and LEUCHTENBERGER using the Millon reaction.

In studying the ratios of substances by comparing the results of two photometric measurements, it has been assumed[145,191] that the nucleic acid

and protein distributions in the cell are similar and that, therefore, the errors due to inhomogeneity will also be similar. In fact the errors will only be the same if the extinctions found by both methods are equal, as this error is also a function of extinction.

4. *Other staining methods*

DANIELLI[71] has suggested the coupling of diazonium hydroxides to specific protein side-chains to give coloured products, which may be estimated photometrically. So far this method has not been used quantitatively.

A semi-quantitative method of estimating diamino acids with acid dyes has been employed by HYDÉN[147]. This is based on the *in vitro* work of CHAPMAN, GREENBERG and SCHMIDT[148].

It would appear that at present those staining methods, which may be considered quantitative, can, under certain conditions, determine the relative amounts of D.N.A, the amount of polymerised D.N.A. and, when used in conjunction with ultraviolet absorption, of R.N.A. by difference measurements.

DISCUSSION

This article has reviewed the physical background to the application of quantitative spectrophotometric measurements to living and fixed cells. Since CASPERSSON[8] demonstrated that spectrophotometry could detect nucleic acids and certain proteins in the quantities in which they occur in the cell, there has been great interest in these methods and their application to problems of growth, cell division and differentiation. A large literature has accumulated during the past sixteen years which cannot be reviewed in detail here. In the case of the ultraviolet work, this has been done by CASPERSSON[9] himself, BRACHET[149] and DAVIDSON[150] among others. Certain past trends will be considered and certain physical and biological problems governing the choice of experiment will be discussed.

It is perhaps necessary to state at the outset that most of the spectrophotometric work of the CASPERSSON school and others was done before ideas regarding the constancy of certain cellular components had been widely considered, following the work of BOIVIN, VENDRELY and VENDRELY[151] and RIS and MIRSKY[152].

CASPERSSON[9] has advanced certain important theories regarding cytoplasmic protein synthesis and gene action which are embodied in his well-known diagrammatic schemes. In describing the changes that occur during mitosis, CASPERSSON has compared meiotic stages in *Gomphocerus* spermatocytes with the interphase nuclei of *Drosophila* salivary glands as he considers that vertebrate cells are less suitable for quantitative measurement.

During prophase, CASPERSSON[153] finds a certain increase in the amount of nucleic acid in the chromosomes and a large decrease in the ratio of proteins to nucleic acid. He has concluded that the chromosomes lose proteins during this stage and that in the reversed process, during telophase, protein accumulates around the 'gene loci'. CASPERSSON has assumed that this is new protein synthesised on the chromosomes during this short period and therefore calls it the 'primary gene product'. There is however, no evidence that the protein appearing is not of extra-chromosomal origin or that it is not the same material as that lost earlier at prophase, which may have been concerned with spindle formation.

The importance of the heterochromatin is further emphasized; CASPERSSON[9] has concluded that it 'directs the nucleic-acid metabolism in the other chromosomes of the nucleus, the nucleolus, and the cytoplasm as well as in the chromosome in which it lies'. This conclusion is the result of work on the salivary gland chromosomes with SCHULTZ,[154–157], in which they found that the genetic alteration of the heterochromatin resulted in alterations in the amounts of nucleic acid in the neighbouring bands, in the nucleotide and protein metabolism in the other chromosomes, the composition of the nucleolus, and the protein and nucleic-acid metabolism of the cytoplasm.

The work on cytoplasmic protein synthesis[9,149] has shown that where there is a high protein-synthesis there is also a well-developed nucleolus and a high concentration of ribose nucleotides in the cytoplasm. Gradients of nucleotides and proteins have been reported by CASPERSSON and his co-workers[158–161] to exist in a few types of cells. They do not appear, at least as far as the nucleotide gradients are concerned, in rapidly growing tissue culture cells[20]. A comparison of the rates of protein synthesis in tissue culture cells and the cells studied by the CASPERSSON school would be among the factors necessary in order to evaluate the weight of this criticism.

These theories of protein synthesis are founded essentially on a static distribution of absorbing materials. BRACHET[149] (p. 233), has commented : 'As a result of this, CASPERSSON's interpretation of the facts becomes hypothetical just at the point when he tries to translate them from a descriptive and topographical level to a physiological one'. Within the limits of the method and materials the CASPERSSON school has sought to overcome this difficulty in various ways. Thus, THORELL[46] has applied a more dynamic approach to the various constituents of blood cells during hematopoiesis, where definable stages in development may be recognised. The work on nerve cells was characterized by the experimental modification of nerve cell metabolism, as well as measurements during various physiological states[161,162]. An extensive series of 'disturbed systems for protein formation', such as carcinomas and sarcomas, were investigated[72].

Yeasts and bacteria, where growth and division may be more easily controlled, have also been studied[163,164].

In general this work has supported the topographical results upon which CASPERSSON's earlier hypotheses are based. But at the same time these earlier results have been utilized to identify the state of the nucleotide-protein synthesis system.

It may be criticized from the technical aspect in that fixed cells have been used; fixed cells have definite advantages but it is not known how much differential contraction or expansion of the morphological components or chemical constituents has occurred. Second, the non-specific light loss, as measured by the apparent absorption at 312 mμ is often very high. Errors due to scatter and other causes may therefore be serious. While these errors exist, the highly reproducible photoelectric methods, while easily distinguishing the shapes of absorption spectra, may not accurately relate the height of the absorption peaks to the number of absorbing molecules in these materials.

These criticisms are important in a method which relies so much on the interpretation of protein-nucleic acid ratios. They do not, however, invalidate results where the recorded nucleotide variations are large, such as those supporting the conclusions regarding cytoplasmic protein synthesis.

There is a general criticism of the presentation of spectrophotometric results which concerns the too facile derivation of the *amount* of protein or nucleic acid from what is, in fact, a highly reproducible absorption spectrum through a small area of the cell which may not be a fair sample of the whole structure. It is only justifiable to compare the quantities of substances in different cells from such measurements of mass per unit area, provided that variations in the projected area are also taken into account. This is not always clear from the published evidence.

An example of the confusion that may occur is furnished by LESLIE and DAVIDSON[165]. They point to the apparent contrast in chick liver between their own results, which show that the average content of R.N.A. phosphorus is the same in the 2-day old chick as in the 8-day embryo, and the results of CASPERSSON[160] and CASPERSSON and THORELL[166] (see also CASPERSSON[9], pp. 111, 112) who showed that the *concentration* of ribose nucleotides was much higher in the cytoplasm of embryonic cells. After ruling out the possibility of CASPERSSON's results being due to large quantities of acid soluble nucleotides in the embryonic cells, LESLIE and DAVIDSON conclude that 'the lower concentrations in the adult cell may be due to the diluting effect of increased protein and not to a decrease in the absolute amount of nucleotide per cell'.

The exact position of the CASPERSSON school with reference to protein synthesis is not at all clear. CASPERSSON[9] (p. 103) states that there is an

increase in cytoplasmic ribose nucleotides during protein synthesis, and it is generally understood (for example by HUGHES[144]) that a greater *amount* of nucleic acid is associated with this process. Yet THORELL[46] (p. 13) is explicit in referring to the published work of the CASPERSSON school: 'an intense protein generation in the cytoplasm is always correlated with an increase in the cytoplasmic *concentration* of ribose nucleic acid' (*our italics*).

It is, in fact, the high prior R.N.A. concentration that THORELL[46] (p. 74) correlates with the later increase of cytoplasmic protein. On the other hand, it is possible to multiply his figures for cell volume and R.N.A. concentration to give a measure of the total amount of R.N.A. The nuclear volume is neglected in this calculation, but it decreases during this period

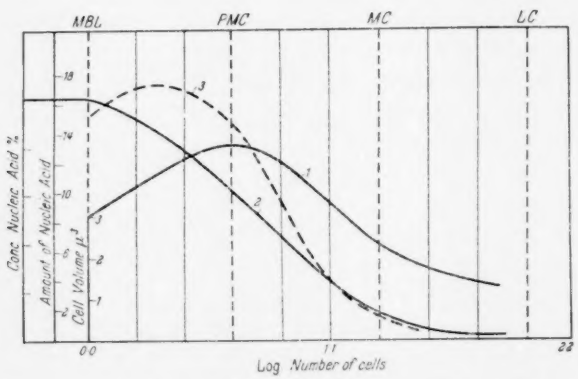


Figure 6. Changes in cell volume (Curve 1) and the concentration of cytoplasmic nucleic acid (Curve 2) that occur during granulo cytopoiesis. MBL = myeloblast; PMC = Promyelocyte; MC = myelocyte; and LC = mature myelocyte. These two curves have been taken from Figure 43 of THORELL'S paper[46] on the formation of cellular substances during blood cell production. Curve 3 is the product of these two curves and represents the total amount of cytoplasmic nucleic acid. The figure illustrates the detailed differences that may be expected when amounts and concentration are compared.

giving a higher proportion of cytoplasm. THORELL'S curves for cell volume and R.N.A. concentration are compared with this roughly calculated measure of total amount in Figure 6 (see CASPERSSON[9], p 145).

However, THORELL considers that the inhomogeneity of the cytoplasm makes it difficult to measure total amounts of R.N.A., a difficulty which must also apply to his measurements of R.N.A. concentration. He then states that both the amount and concentration decrease between the myeloblast (MBL) and promyelocyte (PMC) stages. This confusion illustrates the difficulties that arise when no clear distinction is made between concentrations and total amounts.

Another example in which variations of mass per unit area are given as evidence for variations in total amount is in the measurements of COLE and SUTTON[167], who attempted to contradict results of SCHULTZ and CASPERSSON[154] concerning the amounts of nucleotides in the bands of salivary gland chromosomes on the basis of a single measurement of optical extinction in each band.

It is, however, in the visual estimation of stained specimens and ultraviolet photomicrographs that the most serious errors have occurred. This type of error is important, not only because of its intrinsic magnitude, but because many accepted cytological theories are founded on qualitative estimation. It has been fundamental to much cytological and genetic theory that the amount of D.N.A. in the metaphase chromosomes is much greater than in the interphase nucleus[168–170]. The direct measurements of PASTEELS and LISON[132], and the indirect evidence of SWIFT[69] and of WALKER and YATES[99], would support the view that the qualitative estimations of Feulgen staining are wrong. While photometric measurements on the metaphase chromosomes, such as those attempted by PASTEELS and LISON, may not be very accurate due to the nature of the material, visual estimations must be even less reliable because of subjective errors.

Increasing chromatin stainability has also long been regarded as a characteristic of pycnotic cells. However, LEUCHTENBERGER'S[145] quantitative measurements of the amounts of Feulgen and Methyl green staining show that the total amount of these stains decreases during pycnosis.

In 1946 BRUMBERG and LARIONOW[171,188] reported that living unirradiated tissue culture nuclei did not absorb at 260 mμ, and that continued irradiation resulted in a large increase in absorption. They used achromatic reflecting objectives, focusing with visible light. On this evidence they questioned the validity of results obtained mainly from fixed cells which do show a high specific absorption. Subsequent work, relying on photometric measurements rather than the visual estimation of photomicrographs, has shown that living unirradiated nuclei do in fact absorb[53,172,173], but either mechanical or radiation damage will cause them to round up and shrink with a consequent increase in extinction per unit area, similar to that shown by stained pycnotic cells. RIS and MIRSKY[172] considered that this increase in extinction is due to increased non-specific light loss (scatter) in the damaged cells, while WALKER and DAVIES[53] reported that in chick fibroblasts the decrease in projected area would wholly account for this increase in extinction per unit area and that there was in fact a small decrease of the total absorbing contents of the nucleus.

It is important to distinguish between BRUMBERG and LARIONOW'S error in relying on visual estimation which induced them to believe that nucleic acids in the living state do not absorb, and the salutary reminder that damage due to radiation during exposure or focusing, and to injury during

preparation, may cause considerable changes in the distribution of absorbing material.

Further examples of errors due to visual estimation are given by ALFERT[138], who has discussed in an interesting paper the misleading appearance of some stained cells. He has shown that in primary oocyte nuclei of the mouse, occurring in three classes of mean volumes, 434 μ^3, 1,365 μ^3 and 6,010 μ^3, the amount of D.N.A. as measured by Feulgen staining is the same and that this is the $4n$ quantity. This is in contrast to the often reported negative findings for Feulgen staining in oocytes[174–176]. ALFERT points out that this has been due probably to the concentration of D.N.A. being too low to detect in these large cells and not to the amount being too small. He gives another example from BRACHET[149] who illustrates nuclei of various stages in the development of the sea-urchin embryo and states that 'the affinity of the nuclei for basic dye increases because of the increasing content of thymonucleic acid (D.N.A.)'. However, the illustrations show a parallel increase in dye intensity and decrease in nuclear projected area so that the total amount of stain may remain constant. It is possible that other results, such as those on the 'partial synthesis' of nucleic acid[149] may be open to similar criticism.

The elimination of errors due to the visual estimation of absorption, not only in stained specimens but also in ultraviolet and visible photomicrographs, where paper and film gammas are notoriously misleading, would appear to be one of the most important contributions of quantitative spectrophotometry. Further, the measurement of total amounts will make possible the more accurate comparison of cells under different physiological and experimental conditions, while revealing correlations regarding chemical concentration gradients that are independent of cell volume.

Methods of integrating absorption to give total amounts have been discussed earlier, and used especially for the photometric measurement of Feulgen staining. Relatively little work has so far been done in measuring total ultraviolet absorbing materials. This has been due in part to the difficulty of measuring scatter in fixed cells and also to the laborious techniques formerly available for integrating absorption over a statistically significant number of cells. Special photoelectric scanning techniques[177] and automatic recording densitometers for the photographic method[51,53] have been designed to overcome these difficulties.

Integration measurements have been made for salivary chromosome bands[154], meiotic prophase chromosomes, isolated liver nuclei and sperm nuclei[100], various living sperm, and erythrocyte and tissue culture nuclei[98,99]. As stated earlier, LEUCHTENBERGER *et al.*[100], and WALKER and YATES[98,99] have found in the nuclei of non-dividing cells reasonable agreement between the amount of ultraviolet absorbing materials calculated as nucleic acid and their D.N.A. content as found by bulk biochemical

extractions. WALKER and YATES[99], however, showed that recently divided tissue culture nuclei have a high content of ultraviolet absorbing molecules not accounted for by larger amounts of D.N.A. as measured by Feulgen staining. They found also that ultraviolet absorbing material doubles during interphase.

Thus we have seen that total amounts of substances, their concentration and mass per unit area are among the properties of cells which may be compared by optical means. These properties also include the kinds of chemical compounds present, as identified by absorption curves or staining, the determination of gradients, and the size and shape of the cell. As well as microspectrographic techniques, other methods available are x-ray analysis[178] and cell refractometry[179–181], but they are outside the scope of this article.

While it may be practicable to fulfil all the technical conditions regarding choice of specimen, optics, integration and recording accuracy, there will still be doubts as to the validity of the results, if they cannot be checked, at some stage at least, against similar results obtained by other methods. The most important complementary technique available at present is that of the biochemical analysis of large numbers of cells, with its attendant difficulty of obtaining sufficient quantity of one type of cell only. Thus, for example, the comparison of results on dividing cells is complicated by lack of suitable bulk material with a high mitotic index. Regenerating rat liver[182,183] and ascites tumour cells[192] are among the few suitable materials available. Further, the chemical isolation of dividing nuclei will probably remove labile molecules from the nucleus which may not be distinguishable from similar molecules of cytoplasmic origin. The recently introduced modification of the Behrens isolation technique[80], using non-aqueous solvents, may provide a method of overcoming this latter difficulty.

Results obtained by ultraviolet absorption measurements may also be checked against staining techniques, such as Feulgen or Methyl green, and evidence regarding nucleic acid synthesis may be supported by the autoradiographic demonstration of the uptake of P_{32} by D.N.A.[184]. It may be objected that these corroborative techniques are also subject to criticism, but the ambiguities in each of these methods are of different origin and the errors introduced are likely to differ.

So far we have considered problems of measuring total amounts of substances in cells. It is not sufficient, however, to compare quantities of substances in a well-defined cell structure unless the stage in the cell cycle is known. Thus, CASPERSSON and SCHULTZ[154–157], studying the occurrence of various proteins and nucleic acids in the Drosophila salivary gland nucleus, have postulated an elaborate causal chain stemming from the initial genetically controlled alteration in the heterochromatin. This analysis would be on firmer ground if more information were available

regarding the time relations between the different stages, and indeed, LAGERSTEDT[185] has recently found that in rats fed after starvation the reappearance of heterochromatin and larger nucleoli precedes the reappearance of cytoplasmic R.N.A.

However, information about the history of tissue cells is often difficult to obtain. While the study of certain differentiated cells under physiological or experimental conditions is possible, the study of dividing cells is complicated if the mitotic index, the number of cells preparing to divide, the interphase period, and the time since the last division are not known. An example of the type of error that may be present is shown in SWIFT's work on the D.N.A. content of cells in dividing larval tissues. He deduces from the shape of the histogram relating number of cells to D.N.A. content that synthesis occurs late in interphase. If a proportion only of the cells measured were going to divide, and those that were not remained at the lower level, as has been shown to be the case in tissue cultures, the D.N.A. would increase earlier in interphase.

Tissue cultures would appear to be most useful for the study of dividing cells and undifferentiated growth since not only can they be investigated optically in the living state, but film records over a period prior to the measurement can give the essential time data. They are less convenient for the study of differentiation for although this will occur in tissue cultures[186], it is uncommon to find single layers of flattened cells suitable for optical study.

Knowledge of the time relations between the appearance of nucleic acid and protein production will not in itself provide the key to protein synthesis, as THORELL has shown in his work on blood cells. It is perhaps rather in the finer experimental control of the various processes involved that the advantages of an accurate knowledge of time relations will be most apparent and progress most rapid.

Quantitative cytochemistry involves the study of the relations between chemical molecules and morphological features. It is a borderline subject at the frontier where biochemistry and biology meet. Its present difficulties are largely occasioned by the attempt to apply relatively exact physical techniques to complex biological systems. Its development will depend on the application of new techniques, such as mass determination by cell refractometry, the improvement of instruments, and the selection of suitable types of experimental material. The cytochemist must be aware both of the limitations and the great opportunities of this branch of research.

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8

METHODS OF DETERMINING THE FORM AND DIMENSIONS OF PARTICLES IN SOLUTION: A CRITICAL SURVEY

C. Sadron

I. INTRODUCTION

DURING the last twenty years there has been a considerable intensification of research destined to give us a better knowledge of submicroscopic particles dispersed in the liquid phase. This is, it seems, attributable to two principal causes: in the first place to a rapid development of the industry of high polymers (plastic substances, artificial textiles *etc.* . . .) and in the second to the progress accomplished by biologists. The latter, since the beginning of the century, have indeed succeeded in isolating 'molecular' species of high mass (nucleic acid, proteins, nucleoproteins *etc.* . . .) which constitute the essential materials of which living organisms are composed. In this field they have shown that not only does chemical composition play its part but also the disposition of the functional groups with respect to one another. Hence it is no longer necessary to be restricted to the simple chemical analysis of these elementary particles, but to examine, with the greatest care, qualities other than their internal structure, *e.g.* their shapes and dimensions, as well as certain electrical, optical and mechanical properties. When it is realized that the essential biological mechanisms operate precisely in this region, the importance of this new branch of physical chemistry to biologists will immediately become apparent.

It is reasonable to hope that a detailed study of the morphology of biological macromolecules, by the methods to be described, will permit, for example, the elucidation of the relations between specifically biological phenomena and physical structures, and conversely, enable us to bring about specific changes which promote desired alterations of structure.

THE MACROMOLECULAR DOMAIN

Before entering into the subject fully it will be useful to state some very general considerations about macromolecules.

Firstly, the order of macromolecular dimensions extends from about ten Å to several thousand Å. These limits are evidently very wide. Beside this, there exist certain macromolecules composed of thousands of atoms which are visible to the naked eye *e.g.* the diamond crystal. These cases are, however, exceptional and the largest of the macromolecules to be considered are just below the resolving power of the best optical microscopes, where visual observation is impossible. It must be noted that even the largest of the macromolecules are small enough to be involved in an intense brownian movement. This will be a factor of great importance in all that follows.

The lower limit of the macromolecular domain is still less precise. It is estimated to be in the neighbourhood of some 10 Å for, in general, below this limit the molecules are sufficiently simple for their structures to be determined completely by the usual physical molecular methods (x-ray diffraction, infra-red and Raman spectra).

Although rather vaguely defined in this way, the macromolecular domain has its own definite physical properties.

The spectral methods (from x-rays to the infra-red) which have such power for studying small molecules, lose much of their efficacy in the case under consideration : they give in fact information concerning detail, but not assembly. They indicate how the atoms or certain groups of atoms are located, but they only give very vague indications of the whole assembly which they constitute. The molecular physics of small molecules is powerless to give us the outline, dimensions, shape and internal arrangement of the general structure. If the molecule is no longer a rigid structure, but is flexible and of varying configuration, it is still further powerless. For the study of macromolecules, especially in solution, it is imperative to use specialised techniques and theories, which now constitute a well defined chapter of molecular physics.

THE TWO EXTREME TYPES OF MACROMOLECULES

In the first place, a direct distinction between two extreme types of macromolecules is evident : namely rigid molecules and flexible chain molecules.

Rigid macromolecules consist of an assembly of atoms where the nuclei are situated in perfectly defined positions with respect to each other, from which the vibrations responsible for the infra-red and Raman spectra arise. Such a macromolecule is a small solid of well defined shape *e.g.* a microcrystal.

On the other hand, chain molecules *e.g.* the linear high polymers, consist of successions of atoms, or groups of atoms, united one after the other by the valency links which give a certain number of degrees of freedom. In these conditions, in general, the macromolecule no longer

has definite shape, but presents an infinite number of configurations and, in fact the idea of shape must be replaced by that of a statistical configuration.

It is obvious that a whole series of continuous transitions exists between the extreme possibilities of rigid and chain molecules.

The description of the configuration of a macromolecular chain presents, it will be recognised, difficult statistical problems. Here we shall restrict ourselves to the case of rigid macromolecules whose shapes and dimensions are so well defined that they will convey the impression of a real molecule *i.e.* an aggregate of atoms placed in accordance with the prevailing valency links, or of an ionic crystal or even a micelle. Our problem becomes at this point a problem of colloidal physical chemistry and in future we shall discard the term 'macromolecule' and substitute that of 'particle' or 'colloidal particle'.

The following point may now conveniently be considered. If we are to be interested here only in rigid macromolecules, that presupposes that we can differentiate between a solution of rigid macromolecules and a solution of flexible chain molecules. Often we do know from simple chemical considerations with which category we are dealing. Thus, we know that paraffins of formulae $CH_3 — (CH_2)_n — CH_3$, are chain molecules, and that a diamond crystal is a three dimensional rigid molecule. But it is not always so easy, particularly with particles of biological origin, and appropriately enough, it will be one of the conclusions of the methods to be discussed, to indicate whether the solution contains flexible filaments or rigid particles.

It will become apparent later that such a classification is not easy to operate, and also that, when external considerations cannot be dispensed with, it will be difficult to avoid some confusion between the two categories, which may lead to radically false conclusions about the structures studied. We shall see in the course of the last section how such an unfortunate confusion can be avoided.

STATEMENT OF THE PROBLEM

Suppose then, for the moment, that we are dealing with a solution of rigid particles whose dimensions are within the region described above (*ca.* 10 — 1,000 Å). It is proposed to determine the dimensions of these particles, also their mass, and at the same time, certain of their physical characteristics from which a knowledge of their internal structure can be obtained. Briefly, we propose to measure the characteristic dimensions of the particles in solution.

But it often happens in practice that one ignores initially whether or not the solution contains particles which are all identical.

The problem to be considered consists of sorting the particles into different categories, so that all the particles of each group possess the same shapes and the same dimensions.

Consider a solution containing ν particles per unit volume (in general per cubic cm); it is required to determine the number $\nu_1, \nu_2, \ldots \nu_i \ldots$ of particles per unit volume in the morphological groups 1, 2, . . . i, . . ., or again the percentages $\nu_1', \nu_2' \ldots \nu_i' \ldots$ of these particles contained in any volume of the solution.

A case frequently realised is that where all the particles are geometrically similar. The different morphological groups can then be characterised by a dimension l of the particle, when the problem becomes one of giving the percentages $\nu_1', \ldots \nu_i' \ldots$ of particles corresponding to the values $l_1, \ldots l_i \ldots$ of l. Hence it is necessary to define the function of a characteristic dimension $\nu_i'(l_i)$ which represents the percentage of particles of a given size in the mixture.

When the magnitude of l can be considered as a continuous variable between extreme values l_1 and l_2, the distribution for the percentages of particles in the mixture whose size is between l and $l + dl$ is expressed as :

$$\frac{\rho\,(l)\,d\,l}{\int_{l_1}^{l_n} \rho\,(l)\,d\,l}$$

From this the distribution is known, if the function $\rho(l)$ is known, and is called the density of distribution of the particles according to the dimension l.

For example, it frequently happens that the particles may be rigid rods of the same cross-section, but of different lengths; (solutions of V_2O_5, of the tobacco mosaic virus, and of various 'fibrous' proteins).

This function constitutes then, from all the evidence, the morphological character l, and leads to the experimental determination of the function $\rho(l)$.

Finally it should be noted that if the particles are all of the same kind they all have the same density and consequently the law of distribution according to dimensions follows immediately by a simple calculation from the law of distribution according to mass.

In this simplified form the problem falls into two parts :

(*a*) recognition of the common shape of particles in solution.

(*b*) determination of the distribution function for the particles according to a characteristic dimension (or according to mass).

If the particles do not have a common shape, the problem posed in general terms becomes much more difficult to resolve. In general, it is necessary to effect a separation of the constituents of the solution, but each case is then a special one demanding methods which depend essentially on the peculiarities of the solution under consideration.

THE ELECTRON MICROSCOPE

Before pursuing, in the following chapters, the study of the methods used in the solution of the problem, it is necessary to devote a paragraph to the electron microscope.

This instrument reveals, after suitable preparation, particles whose dimensions are largely below 1,000Å. As is well known, the resolving power of electron microscopes descends, at least theoretically, and in practice for the best instruments, to a limit below about 20Å. In these conditions the macromolecular domain will be almost entirely within the working limits of the electron microscope, and the problem described above will be resolved automatically. It suffices to replace the traditional "optical" microscope by the more modern electron microscope. As a result of this, an immediate observation of the shapes of the particles studied is often possible, frequently by means of the shadow method. Further micrometric examination of a sufficient number of photographs of particles allows the deduction of the law of distribution ν_i' in groups of homogeneous shape and size.

Briefly, the use of the electron microscope seems to solve completely the problem stated in the preceding paragraph.

Certainly the electron microscope affords an excellent method for studying the morphology of particles. But without underestimating its importance it should be emphasized that this method is, as with all experimental methods, open to criticism, and suffers limitations which make other methods necessary.

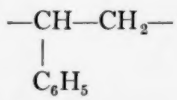
The criticisms may be stated briefly: we shall confine ourselves to those which are of greatest importance for the subject:

1. It frequently happens that, whilst one of the dimensions of the particles may be very much too big for the limit of resolution of the electron microscope, the others are too low. For this category of particles the electron microscope is useless. This is the case, for example, with particles which have the form of very thin rods: *e.g.* molecules of thymonucleic acid are invisible whilst often having lengths greater than 3-4,000 Å; this invisibility is due without doubt to their thickness being less than about 15 Å. This is also the case with some fibrous proteins, as well as for most rigid chain molecules, such as the nitrocelluloses.

A similar situation occurs with certain globular proteins such as the albumens and the globulins.

2. In the favourable cases, where the particles are plainly visible, an extremely important question arises: do the shapes and dimensions of the photographed particles correspond exactly to the shapes and dimensions of the particles in solution? It is known in fact that the particles are deposited on the preparation frame after desiccation of a small drop of the solution, after which they can be silhouetted by thin metallic films as in the shadow method, or rendered opaque by impregnation with a metallic salt. This treatment may modify the particle and the least that can be said is that the electron microscope gives the photograph of a type of macromolecular skeleton which is perhaps as different from the free molecule in solution as is the dried plant from the same plant when growing naturally.

That is perhaps appropriately illustrated with the example of polystyrene. The molecules of these high polymers consist of repetitions of the group:



and not only the structure, but also many observations of their solutions have led to the assumption that they are definitely chain molecules, that is to say, filaments made up of many winding configurations.

However the electron image of the macromolecules deposited on the film carrier after evaporation of a solution in cyclohexane, is exactly spherical, as is shown in Figure 1. Perhaps this example constitutes an extreme case, but it shows clearly that the electron image alone is not sufficient to determine with certainty the shape of a particle in solution. In the case of rigid particles, modification brought about by desiccation is perhaps less considerable, but it remains a possibility that the observed dimensions are false. It will be necessary to return to this important point in detail later.

3. Finally—and with the reservations made above—the electron microscope gives nothing but the shape of the particles, and affords no information concerning their internal properties. An advantage of the methods to be described is that they also give other information *e.g.* certain electrical properties of the substance, such as polarisability, a property which, it is hoped, can be related to the atomic structure of the particle.

From this brief analysis it will be concluded that the use of the electron microscope, although of wide application for the study of the morphology of particles in solution, does not eliminate in any way the necessity of the use of methods in which the particles are studied in the liquid medium. These methods will now be described.

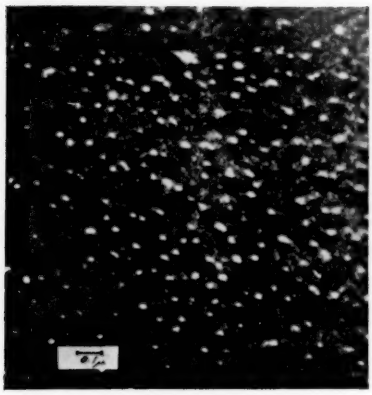


Figure 1. Chain molecules (polystyrol) appearing as spherical particles in electron microscope (from SIEGEL, JOHNSON and MARK[1]).

II. METHODS OF MEASUREMENT IN SOLUTION

1. GENERAL PRINCIPLES.

Contrary to the electron microscope method, where measurements are made on individual particles, the methods now to be discussed are concerned with a mean property of a macroscopic quantity of the solution, and therefore relate to a large number of particles. Having measured the mean property it is necessary to establish :

1. The relation between the mean observed macroscopic observation and the size of each elementary microscopic phenomenon, relative to each of the particles contained in the mass of the solution submitted to experiment.

2. The relation between the magnitude of the microscopic phenomenon and the dimensions of the particle.

These two relations, established by means of a suitable theory, permit the required sizes to be calculated from the results of observation, but when thoroughly applied impose serious handicaps.

1. The calculation is only possible when the elementary property relative to each particle which produces the measured effect is independent of the presence of other particles. For example, the energy dissipated by viscosity when a particle moves relative to its 'solvent' can easily be calculated for a single particle in the fluid *i.e.* if the concentration of the solution is practically zero. Otherwise the calculation is extremely difficult and can only lead to a result in very simple cases.

As before let ν be the number of particles per cc and ϕ be the magnitude of the elementary phenomenon for which each particle is responsible (energy dissipated by viscous flow, intensity of diffracted light, *etc.*), and Φ be the overall phenomenon observed in the assembly of the particles.

In general ϕ depends on ν and we can write :

$$\phi = \phi_0 + a\nu + b\nu^2 + \ldots .$$

where ϕ_0 represents the magnitude of the elementary phenomena at zero concentration—*i.e.* when the particle is alone in the fluid. Further we have :

$$\Phi = \nu\,\phi_0 + a'\nu^2 + b'\,\nu^3 + \ldots .$$

Hence by measuring Φ for decreasing values of ν, the limiting value of Φ/ν for $\nu = 0$ gives ϕ_0 *i.e.* the magnitude of the elementary phenomenon, of which the theory permits calculation from the dimensions of the particle.

A suitable extrapolation thus gives ϕ_0 from Φ.

The methods used must therefore be designed to find Φ/ν for such small concentrations that the extrapolation towards $\nu = 0$ can be made

with sufficient accuracy. This justifies the study of macromolecular solutions at very high dilutions.

It is evident that at large dilutions the magnitude of the overall phenomenon is very small, so that it is necessary to use very sensitive techniques.

2. Having thus determined the magnitude of the elementary phenomenon ϕ_0 it can be expressed as a function of the characteristics of the particle *e.g.* their dimensions.

Whether the phenomenon ϕ_0 is hydrodynamic friction coefficient, or the dispersion of an incident electromagnetic field, it is clear that the calculation will only lead to a result when each elementary particle has a simple geometrical shape.

In fact, at present these calculations are only possible if the particle is close to a sphere, a very elongated or flattened cylinder of revolution, an ellipsoid of revolution, or strictly a scalene ellipsoid.

It follows that the methods in solution cannot pretend to furnish an exact description of the shape of the particles and that they will only give the dimensions of the shape of revolution which approaches the real shape. This is undoubtedly a drawback of this type of method, but its importance must not be exaggerated : the real shape of macromolecules in solution is in fact so far from clear that the primary consideration is to establish the symmetries and the characteristic dimensions of the particles.

Finally it is necessary to recall that in the above analysis we have put $\Phi_{\nu=0} = \nu\phi_0$ which supposes that the phenomenon ϕ_0 is the same for all the particles contained by the solution. This assumes implicitly that all the particles are identical (solution monodisperse).

Particular methods will be described in the following chapter which are actually used on the assumption of the following three conditions :

(*i*) the solutions are infinitely dilute.

(*ii*) they contain particles of simple geometric shape (solids of revolution).

(*iii*) they are monodisperse.

It has been explained above that the first two hypotheses were not really restrictive and it has been shown that they do not present serious difficulty in their application to real solutions.

The third hypothesis, on the contrary, is in general inadmissible. Monodisperse media are rare except amongst those of biological origin. A description and discussion of monodisperse media follows, principally to clarify this account and because methods applicable to polydisperse media rely on methods valid for monodisperse media to which they are not in principle dissimilar. The difficulties appear when actual values are interpreted and, as will be seen, these difficulties are still far from being overcome in a satisfactory manner. In fact, the determination of the

distribution functions defined in the preceding pages is one of the pressing problems to which the last paragraph of this chapter will be devoted.

Naturally the methods in solution will be classified according to the nature of the phenomenon ϕ of which the overall effect is observed and which depends on the sizes of the particles. There are two principal groups of methods. In the one, the elementary phenomenon consists of the viscous force exerted on the particle, moving relatively to the surrounding medium; in the other group, the elementary phenomenon consists of the effect of each particle on electromagnetic waves in the solvent (electromagnetic, or optical methods).

2. HYDRODYNAMIC METHODS.

A. COEFFICIENT OF LAMINAR FLOW

(*a*) *General definitions*

Consider a solid of any dimensions moving in a continuous fluid. The fluid, which is assumed to be initially at rest, is disturbed by the passage of the solid so that to maintain the latter in motion an external agency must furnish certain work, in other words the fluid exerts a friction on the solid.

Hydrodynamics shows by theory and experiment that if the velocity of the solid relative to the liquid initially at rest is less than a certain value the disturbed layers of the fluid slide over each other like smooth skins, and without turbulent motion. In this case, known as laminar flow, the frictional forces exerted by the fluid on the solid are proportional to the coefficient of viscosity of the fluid, η_0, and to the velocity of the solid. The coefficient of proportionality depends on the shape of the solid.

Consider a sphere of radius R, and volume V, with a uniform velocity of translation u: the resultant of the laminar forces, or viscosity, which it experiences, is a vector applied at its centre O, opposed to the direction of motion, of magnitude:

$$\phi = u f \text{, where } f = 6 \pi \eta_0 R = \frac{6\pi}{1{\cdot}612} \eta_0 V^{\frac{1}{3}} \qquad \ldots . \ (2{\cdot}1)$$

These two equations constitute STOKE's law.

Similarly, if the sphere turns about a diameter with constant angular velocity ω, the viscous forces reduce to a resultant couple Γ which opposes the rotation. Hence:

$$\Gamma = \omega C \text{, where } C = 8V \eta_0 \qquad \ldots . \ (2{\cdot}2)$$

When the sphere possesses both translational and rotational motion it can be shown that the frictional forces reduce to those just given, with the same values for the coefficients f and C, augmented by terms of the

second order dependant on the velocity. Usually it is supposed, as in the case under consideration, that the velocities are sufficiently small for these terms to be negligible.

In the general case where the moving solid is no longer spherically symmetrical the general laws expressed by equations (2·1) and (2·2) are still valid, but the value of the coefficient f depends on the orientation of the solid with respect to its direction of translation, and similarly, the coefficient C depends on the axis about which rotation occurs.

For example, if the solid is an ellipsoid of revolution of which a is the semi-axis of revolution, b is the equatorial radius, $p = a/b$ is the axial ratio, $V = 4\pi b^2 a/3$ is the volume, then the coefficients of friction are functions of p and V for which an expression will be found in the appendix.

In the case of the solid having the maximum dimensions of a submicroscopic particle in solution, it is possible to confirm that the movement of the disturbed fluid is laminar. In fact the critical velocity above which turbulence occurs becomes larger, as the dimensions of the solid decrease. It can be calculated for example, that for a sphere of 1 cm radius moving in water the critical point where turbulance sets in will be reached at a velocity of translation of the order of a few centimeters per second. Consequently for a sphere of 100 Å radius, this value will be several hundreds of metres per second. It will be seen later that the displacements usually obtained do not exceed the order of one millimeter per second. It follows therefore that in the macromolecular domain, only laminar flow need be considered.

Under these conditions, provided that the laws of hydrodynamics for continuous fluids are valid in the macromolecular region, measurement of one of the coefficients f or C for a sphere permits calculation of R, η_0 being known, and, for an ellipsoid of revolution, measurement of both f and C permits calculation of the two unknowns V and p (or a and b).

(*b*) *Brownian movement*

It is obvious that in the macromolecular region the assumption of the continuity of the dispersive liquid is not admissible a priori since the particle undergoes a Brownian movement, which adds a complication, and throws doubt upon the validity of the method just described.

Briefly, the laws of Brownian movement are as follows.

1. The first is strictly statistical and relies on the assumption that, at two instants t and τ, sufficiently separated, the directions of jumps of translation (or rotation) of the particle have independent probabilities.

That implies that the mean square $\overline{l^2}$ of the displacements of the centre of gravity of the particle, measured after equal intervals of time t, is exactly proportional to the time t. The same applies to the mean square $\overline{s^2}$, of the angles which a fixed axis in the particle makes with a fixed direction.

The laws may be stated as :—

$$\overline{l}^2 = 6\,\Delta\,t \qquad \ldots\ldots (2{\cdot}3)$$

$$^2 = 4\,D\,t \qquad \ldots\ldots (2{\cdot}4)$$

The factors Δ and D which measure in some way the intensity of the Brownian movement are called respectively the translational and rotational diffusion constants of the particle in the liquid.

2. The second is mechanical, relying on the theorem of the equipartition of energy, which shows that the constants Δ and D are given by the equations.

$$\Delta = kT/f \qquad \ldots\ldots (2{\cdot}5)$$

$$D = k\,T/C \qquad \ldots\ldots (2{\cdot}6)$$

where T is the absolute temperature of the solution, k is BOLTZMANN'S constant ($k = 1{\cdot}38 \times 10^{-16}$), and f and C are respectively the coefficients of friction of translation and rotation experienced by the particle in the elementary jumps, the succession of which constitutes the Brownian movement.

3. These general laws having been stated, consider a particle in the solution, spherical for example, to experience a force ϕ of constant magnitude and direction. In the absence of Brownian movement this particle will rapidly assume a uniform velocity u, which is reached when the force due to viscosity $u\,f$ becomes equal to the motive force ϕ. Hence :

$$f = \phi/u \qquad \ldots\ldots (2{\cdot}7)$$

If Brownian movement is taken into consideration the velocity of the particle is no longer constant in magnitude and direction : it varies irregularly, but it can be shown to be purely and simply a superposition of Brownian movement and of the movement of translation. The velocity $\mathbf{U}$ of the sphere is then equal to the sum of the velocities $\mathbf{u}$ which would have been observed if no Brownian movement had existed, and of the velocity $\mathbf{u}_s$ produced by the Brownian movement. At any instant : $\mathbf{U} = \mathbf{u} + \mathbf{u}_s$.

Hence if the velocity $\mathbf{U}$ is measured at different instants and the mean result is taken it is found that, since the mean value of $\mathbf{u}_s$ is zero :

$$\overline{\mathbf{U}} = \phi/f \qquad \ldots\ldots (2{\cdot}7^1)$$

which is an analogous equation to (2·7).

If the particle is not symmetrical, the preceding reasoning must be modified. In fact, the Brownian movement of rotation causes the particle to take different orientations according to the direction of translation and, consequently, the coefficient of viscosity which obtains is a mean of all

the values assumed by the coefficient of viscosity for all the orientations of the particle. For that reason it is designated by the symbol $\bar{f}$.

It is the coefficient $\bar{f}$ which appears in equation (2·5) giving the translational diffusion constant. It is given by the following equation :

$$\bar{f} = \frac{6\pi}{1{\cdot}612}\ \eta_0\ V^{\frac{1}{3}} \cdot 1/t(p) \qquad \ldots . \ (2{\cdot}8)$$

where $t\ (p)$ is a function of axial ratio, given in the appendix.
For $p = 1$, $t\ (p) = 1$, and $\bar{f}$ is equal to the coefficient of viscosity of a sphere of volume V. It follows that $1/t\ (p)$ corresponds to what SVEDBERG called the factor of dissymmetry of the particle.

Finally, the Brownian movement of translation has not, after what has been said above, any influence on the Brownian movement of rotation. It is for this reason that the coefficient C of (2·6) is not a mean value.

It has already been stated that, for a sphere turning about one of its diameters, $C = 6\ \eta_0\ V$.
In the case of an ellipsoid of revolution turning about its equatorial diameter, C can be expressed in the same way as $\bar{f}$, as follows :

$$C = 6\ \eta_0\ V \cdot 1/r(p) \qquad \ldots . \ (2{\cdot}9)$$

where $r\ (p)$ is a function of elongation to be found in the appendix. $r\ (p) = 1$ for $p = 1$.

4. It appears then that the existence of Brownian movement does not influence the definitions of the coefficients of viscosity for submicroscopic particles to any noticeable extent, any more than do their sizes. However, one question remains : is it true, as has been assumed, that the expressions for the coefficients of viscosity are still given in this case by the laws of hydrodynamics for continuous fluids?

The answer to this question is not simple and categoric. It is evident that when the dimensions of the particles are very large by comparison with the molecular dimensions of the solvent, the latter can be considered as a continuous fluid and the corresponding laws should be valid. But it is equally probable that, as the particles become smaller and smaller, these laws become less and less exact : the idea of viscosity becomes difficult to define in the molecular region and the problem to be considered is a problem of the kinetics of liquids which has not yet been given a satisfactory theoretical solution.

For the present it will be assumed that in the macromolecular region, the laws of hydrodynamics of continuous fluids are still valid in the mean, and that they give the correct expressions for the coefficients of viscosity. This important point will be considered again in Section III.

(*c*) *Measurement of coefficients of friction*—The methods of determining the radius of a sphere, or the dimensions of an ellipsoid (or a cylinder) of revolution from the coefficients of friction $\bar{f}$ and C are governed by the above considerations. It is now convenient to review the methods for measuring the coefficients. They fall into two groups according to the considerations just made.

The first group includes methods relying on Δ and D, and the application of equations (2·5) and (2·6).

The second includes those relying on the measurement of the mean velocity of displacement, u, of the particle in a uniform field of force g, and equation (2·7).

B. MEASUREMENT OF THE DIFFUSION CONSTANTS

The principle of these measurements is the following:

(*a*) *Constant of translational diffusion*

The solution, of concentration γ, is placed in a vessel of sufficient depth in contact with the solvent. In these conditions the macromolecules diffuse upwards and, after a certain time t, the concentration at a distance x from the surface of separation is $\nu\ (x, t)$. Now it can be shown from the laws of Brownian movement that the variation of the concentration obeys FICK'S law *viz.*:—

$$\frac{\delta \nu}{\delta t} - \Delta \cdot \frac{\delta^2 \nu}{\delta x^2} = 0 \qquad \ldots\ (2{\cdot}10)$$

By integration and from the experimental boundary conditions we have:

$$\nu\ (x, t) = \gamma\ (\lambda - f\ (x/\sqrt{t \cdot \Delta}) \qquad \ldots\ (2{\cdot}11)$$

where f is the Gauss function defined as:

$$f(y) = \frac{2}{\sqrt{\pi}} \int_0^y e^{-u^2} \cdot du$$

The value of Δ can be obtained by comparison of equations (2·11) with the experimentally determined function $\nu\ (x, t)$.

Further it is necessary to note that, in keeping with what has already been said, equations (2·11) are only valid for infinitely small values of γ. It will therefore be necessary to repeat the experiment several times with decreasing initial concentrations of γ, and which give a different value of Δ each time. The value of Δ taken as being correct will then be the value for $\gamma = 0$, obtained by extrapolation.

Numerous techniques allow experimental realisation of the above scheme.

(*b*) *Diffusion constant of rotation* : *D*

The method of measurement can be the same as for Δ, starting from a solution in which the particles are initially orientated, in the mean, in a definite direction and neglecting the cause of the orientation, Brownian movement will progressively restore disorder. The law according to which the mean orientation decays can be established in spherical coordinates from FICK's law*. By comparison with the experimental law the numerical value of *D* can be found.

This is BENOIT's method. The orientation is obtained by the application of an electric field, and it is measured by the birefringence effect produced (Kerr effect). When the field is switched on suddenly the birefringence grows gradually, and then decays when it is switched off (Figure 2).

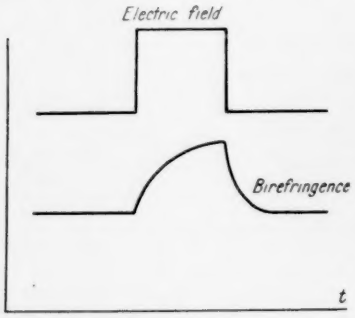


Figure 2a.
Scheme showing application of electric field in Kerr cell and the birefringence observed.

Figure 2b.
Oscillogram obtained with nucleic acid solution of concentration 0·25 g/cm³. (Field applied for several thousands of a second).

The value of *D* can be found from the curves of growth and decay of the birefringence, which can be seen on the oscillograph screen. Experiment gives in addition the value of the permanent dipole of the particles. This method is not applicable to conducting solutions.

A second method, of very general interest is based on the phenomenon of birefringence of flow. It can be shown that non spherical particles (for example, rods or discs, elongated or flattened ellipsoids of revolution) tend to orientate themselves parallel to a given direction when placed in a liquid undergoing streamline flow of constant velocity gradient. For example the solution can fill the interval between two coaxial cylinders (Figure 3a). When one turns with an angular velocity Ω, and if *R* is the radius of the rotor, *d* the distance of separation between the two cylinders, then the velocity gradient *G* is practically constant throughout the liquid

*There is no mention here of the experiments appertaining to these laws because they are irrelevant to the text : the reader who is interested in these questions should consult the general memoirs indicated in the literature at the end of this account.

and is equal to $\Omega R/d$ (Figure 3b). If the particles contained by the solution have a mean orientation, the latter will be birefringent. Plane polarised light crossing the liquid parallel to the axis of the cylinders is elliptically polarised on emergence, except when the incident vibration is parallel with one or other of the two normal directions L_1 and L_2 between them,

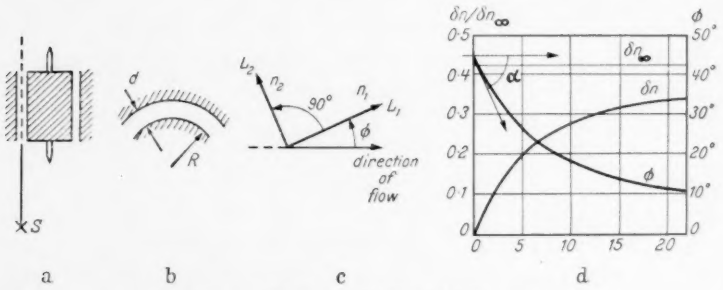


Figure 3. Diagrams of flow birefringence.
(a) Schematic arrangement: S, beam of linearly polarized light.
(d) Variations of φ and $\delta n/\delta n_\infty$ as function of G/D, after PETERLIN and STEWART[3].

called the lines of isocline (Figure 3c). In this case the vibration remains rectilinear but the corresponding indices of refraction n_1 and n_2 are different.

The orientation effect opposed by the Brownian diffusion of rotation increases with the velocity gradient, as does the observed birefringence, which becomes constant when the orientation is complete. But what is remarkable about the phenomenon is that the position of the lines of isocline L_1 and L_2 depend on the rate of orientation. When the orientation is complete (G infinite) all the particles lie along the lines of flow of the liquid, and one of the lines, L_1 for example, is parallel to the flow. When G diminishes L_1 makes an angle φ with the direction of flow, becoming larger and larger and tending to 45° when G tends to zero. The theory of the phenomenon[3] permits deduction of the relation that the tangent of the angle α shown in Figure 3d is given by the relation:

$$\text{Tg } \alpha = 1/12\, D \qquad \ldots . \ (2{\cdot}12)$$

The variation of ϕ as a function of G can easily be followed with suitable apparatus and the corresponding graph obtained. Then the value of tg α for $G = 0$ gives the value of D.

The method can obviously be applied to electrically conducting solutions.

There is also a supplementary advantage; it shows in fact that if the particles can simulate ellipsoids of revolution of axial ratio p and of

volume V, the value of the birefringence $\delta n = n_1 - n_2$ is given by the equation :

$$\delta n = \frac{2\pi}{15} \cdot \frac{e}{n_0} (g_1 - g_2) \frac{V}{M} c \cdot \frac{G}{D} \qquad \ldots . (2{\cdot}13)$$

where e is $\dfrac{p^2 - 1}{p^2 + 1}$

c is the concentration of gm per cc, M is the mass of the ellipsoid, and g_1 and g_2 are two factors given by the equation.

$$g_i^{\varphi} = (n_i^2 - n_0^2) \,/\, [4\pi + (n_1^2 - n_0^2) L_i / n_0^2], \; i = \begin{cases} 1 \\ 2 \end{cases} \qquad \ldots . (2{\cdot}13^1)$$

In this new expression n_0 is the index of refraction of the solvent, and n_1 and n_2 are the principal indices of the ellipsoid, L_1 and L_2 the factors due to MAXWELL depending uniquely on the dimensions of the ellipsoid (see the appendix). When the latter is very elongated for example, the factors take the very simple values :

$$L_1 = 0, \; L_2 = 2\pi$$

Therefore, knowing D (from tg α), and also M/V, the value of $\delta n / cG$, the MAXWELL constant of the liquid, is a known function of n_1 and n_2.

Further, the index n of the solution is also a known function of n_1 and n_2 given by :

$$n = n_0 + \frac{2\pi}{n_0} (g_1 + 2\, g_2) \frac{cV}{3M} \qquad \ldots . (2{\cdot}14)$$

It is clear that n_1 and n_2 can be calculated from values of n and δn *i.e.* the magnitudes related to the internal structure of the ellipsoid. In this manner the method gives not only the shapes and dimensions of the particles, but also the principal features of their construction[4].

C. METHODS OF GENERAL MOVEMENT

These methods differ according to the nature of the forces used to obtain the displacement of the particle in the midst of the surrounding liquid.

The migration of charged particles in an electric field (electrophoresis or cataphoresis) is only used with difficulty for measuring dimensions, since it is necessary to know the electric charge for each particle and generally this is not known.

Another well known method is sedimentation in an intense gravitational field, such as is produced by an ultracentrifuge. The velocity of sedimentation is given by

$$\mu_s = M (1 - V_{sp} \cdot \rho_0) \, g / f \qquad \ldots . (2{\cdot}15)$$

where V_{sp} is the partial specific volume of the particle and ρ_0 the density of the solvent, g the gravitational field, and $\bar{f}$ the coefficient of frictional resistance (2·7[1]).

D. VISCOSITY OF EXTENDED MACROMOLECULAR SOLUTIONS

Whilst a liquid is maintained in streamline flow, it exerts on the walls of the containing vessel forces proportional to its coefficient of viscosity. The coefficient of viscosity can be determined from the measurement of these forces. For example, the determination of the pressure gradient necessary to make the liquid flow in a capillary of given radius for a given rate of flow, or that of the viscous couple exerted on one of the cylinders in Couette's vessel, are classical procedures of viscometry, of which the technique is too well known to be discussed at length here.

If η_0 be the viscosity of a pure liquid, introduce a solid particle into the liquid maintained in viscous flow. The flow is disturbed; supplementary viscous forces act on the surface of the particle and because of this, the viscosity is increased. If η is the viscosity of a solution containing a single particle, then :

$$(\eta - \eta_0)/\eta_0 = a$$

where a is a factor depending solely on the shape and the dimensions of the immersed particle.

It represents the quantity ϕ_0 given in the introduction to this chapter.

If now a solution containing ν particles per cc is considered, the disturbances produced by each particle will be additive, purely and simply, if ν is sufficiently small for there to be no interaction. Hence :

$$\eta_{sp} = a\nu$$

In general, however, interactions occur and hence :

$$\eta_{sp} = a\nu + b\nu^2 + c\nu^3 \ldots .$$

If M be the mass of the particles and c the concentration in gm per cc, $\nu = c/M$, and

$$\eta_{sp} = \frac{a}{M} \cdot c + \frac{b\varphi}{M^2} c^2 + \ldots .$$

The limiting value of specific viscosity for $c = 0$ is called the intrinsic viscosity of the solution $[\eta]$. Then : $[\eta] = \dfrac{a}{M}$

Now the value of a can be calculated from the laws of hydrodynamics for particles of simple geometrical shape*.
For example, for spherical particles of volume V:

$$a = 2{\cdot}5\ V \text{ or } [\eta] = 2{\cdot}5\ V/M \text{ (EINSTEIN's formula)} \quad \ldots . \ (2{\cdot}16)$$

For an ellipsoid of volume V and of axial ratio p ($= \frac{a}{b}$):

$$[\eta] = \frac{V}{M} A(p) \quad \ldots . \ (2{\cdot}17)$$

where $A\ (p)$ is an increasing function of p, equal to 2·5 for $p = 1$ and for which the expression and values are to be found in the appendix.

Here it is convenient to remark that, as already explained, in hydrodynamic flow elongated particles tend to orientate themselves in the direction of flow of the liquid, doing so more easily as their constant of rotational diffusion is the smaller. In these conditions the distribution of the orientation of the ellipsoids in the viscous flow depends on the velocity gradient of the flow. It will be the same for the hydrodynamic perturbation which they produce. Consequently the measured viscosity, η, will depend, in this case, on the velocity of viscous flow. There is said to be an 'effect of orientation' on the viscosity, or alternatively, that the liquid has a 'structural viscosity'. It is necessary to take care, in this case, to make the measurements under conditions such that the velocity gradients in the viscometer may be sufficiently small for the orientation effect to be negligible. This is very easy in practice and it is only in exceptional cases that such effects give trouble. If the viscous flow has a uniform gradient, as in the case of the Couette cell, W. and H. KUHN[7] have established that the intrinsic viscosity $[\eta]_G$ for a small gradient G is related to the viscosity $[\eta]$ of zero gradient, in the case of a very elongated ellipsoid, by the relation :

$$[\eta]_G = [\eta]\ [1 - 1{\cdot}09\ G^2/32\ D^2] \quad \ldots . \ (2{\cdot}18)$$

where D is, as is usual, the constant of rotational diffusion of the particle about an equatorial diameter.

E. PRINCIPLE OF THE MEASUREMENTS OF DIMENSIONS BY HYDRODYNAMIC METHODS

In conclusion, if it is supposed that the particles in solution resemble ellipsoids of revolution—very elongated or flattened—the following magnitudes, which are functions of their volume V and their axial ratio p, and also their mass M, can be determined from experiment:

* See general literature.

1. $f(V,p) = kT/\Delta$ determined from the measurement of Δ.
2. $C(V,p) = kT/D$ determined from the measurement of D (Kerr effect, birefringence of flow, viscosity of structure).
3. $\frac{1}{f} M (1 - \frac{V}{M} \rho_0) = S$ determined by sedimentation.
4. $\frac{V}{M} A(p) = [\eta]$ determined by viscometry.

p and V can thus be calculated directly from the measurement of f and C, independently of M.

The measurements of f or of C (preferably the former, for the constant of rotational diffusion, is sometimes very difficult to measure) can be equated to that of the constant of sedimentation or to the intrinsic viscosity. If M is also known, the two measurements give V and p.

Finally, the measurement of three of the four expressions gives V, p and M.

Thus, in principle, hydrodynamic methods alone can give the mass and the dimensions of the particles.

Meanwhile it is necessary to add immediately that M can be measured directly by other methods, as for example by osmosis or the scattering of light, and that the specific partial volume $V_{sp} = V/M$ for the particles can be determined directly from a simple comparison of the densities of the solution and of the solvent.

In these conditions, in fact always, much that is available to solve the problem is disregarded, and the experimenter can choose what may be easiest to determine in the particular case in question.

From this point of view, it might seem therefore that the problem of the determination of the masses and dimensions of particles in a monodisperse solution is largely resolved and all that remains is to perfect the technique of measurement.

Unfortunately this is not so, as will be shown by the following remarks :

(*a*) Firstly, the functions $t(p)$ and $r(p)$ in the expressions for $\bar{f}$ and C are different according as to whether the ellipsoid is flat or long. Determination of the dimensions of the particle which are based only on the measurement of $\bar{f}$ and C (whether or not involving M, or V/M from other methods) leads to ambiguity.

This is often eliminated because it is known, from consideration of chemical structure, whether the particle is most probably simply a rod or a flat disc.

It can also be eliminated, quite rigorously, in the following way : $\bar{f}$, C and M are measured on the one hand and $[\eta]$ on the other. The values of p, V and M given by the first set of measurements must, since they occur

in the expression $(V/M)\,\Lambda\,(p)$, give the latter a numerical value comparable to that of the intrinsic viscosity. It is clear that this coincidence is unlikely if the model chosen for the ellipsoid is false.

(*b*) It has already been indicated in the general principles at the head of this chapter, that we might suppose the particle were similar to an ellipsoid or cylinder of revolution. Hydrodynamic methods then only give the volume and the axial ratio of the ellipsoid which is equivalent to the real particle in the solution.

This is an inconvenience inherent in these methods. But that being admitted it is permissible to ask whether the values of V, p, M, M/V and V_{sp} measured by diverse techniques correspond to a great extent with the same equivalent particle.

Firstly it is evident that all the above analysis is rigorously correct if the particle in suspension is a small ellipsoid around which the molecules of the solvent circulate freely.

But this case is fictitious. The atoms or groups of atoms at the surface of the particle exert on the molecules of the solvent forces whose intensities depend on the chemical nature of the solute and the solvent. Moreover, the fact of having replaced a volume of the solvent equal to that of the particle by a volume of different material causes the molecules of the solvent in the neighbourhood of the particle to experience a field of force of the type producing surface tension.

These effects give rise to a layer of variable thickness around the dissolved particle, where the molecules of the solvent are either fixed to the particle or partially connected to it, so that the viscosity of the solvent were no longer equal to η_0. Thus the situation is modified as though the particle whose movement is being followed, were surrounded by a solvate film which increased its volume and modified its axial ratio*.

It is quite evident that the coefficients of $\bar{f}$ and C relate to this particular complex, which for this reason will be called the hydrodynamic particle.

It is equally reasonable to admit that the values of V and of p occurring in the expression for the viscosity relate to the same hydrodynamic particle.

As for the mass M, which figures in the expression for the viscosity, this is introduced by writing that the number of particles per cc is equal to c/M. M is then the mass of the 'dry' particle of matter dispersed in the liquid, and is not in fact the mass of the 'hydrodynamic' particle. This value for the mass M will be given more directly, perhaps, by the measurement of osmotic pressure, or of the scattering of light.

*The discussion of the case where the small ions are connected to the macromolecule, causing an increase of viscosity will be left here. It may be that in the course of movement of the particle relative to the solvent the layer of ions might deform and produce an electro-viscous effect. (8)

Consequently the use of the values of Δ, D and $[\eta]$, or the use of two of these values and a value for the mass (*e.g.* from osmotic pressure), leads to coherent results which give the dimensions of the hydrodynamic particle as well as the mass of the dry particle.

Besides, the pycnometric measurement of partial specific volume V/M gives the relationship between the volume of the dispersed matter and its mass (assuming the density of the enveloping dissolved film to be the same as that of the solvent, which it is not at all certain to be). V here is then the volume of the dry particle, to be denoted by V^1. If in addition the value of M has been obtained, pycnometry gives V^1. From values of V and V^1, the volume of the solvent $V - V^1$ associated with the dry particle may be deduced. Yet it is necessary to realize that the volume $V - V^1$ is not necessarily that of the molecules of the solvent actually attached by adsorption to the dry particle, since the particle may have a lacuna structure like a sponge: in this case the solvent molecules circulating in the hollows are incorporated in the general movement of the particle without adsorption necessarily actually occurring.

It is usual, particularly in biological work, not to use the sets of measurements which have just been discussed, but rather those including $\bar{f}$ obtained by sedimentation, Δ (often obtained in the course of the same experiment), and lastly that of V^1/M from pycnometry.

It will be noticed first that the ratio V^1/M appears, in the expression (1·3) for s, which is equal to V_{sp} and which is found from a simple pycnometric measurement, subject to the condition that the dissolved layer has a density equal to that of the solvent. Combination of the expression for Δ (2·1) and that for s (2·3) (see p. 255) gives:

$$S = M \frac{(1 - V_{sp}\,\rho_0)\;\Delta}{kT}$$

where the values of S, Δ and V_{sp} are obtained experimentally. This expression allows calculation of the value of M, then, knowing M, formula (2·15) gives $\bar{f}$.

When no solvation occurs, the volume V of the particle is given by the relations: $V = V^1 = M\,V_{sp}$ and its axial ratio is obtained from the expression for $\bar{f}$, (2·8), which also gives the value of t (p), V being known.

When solvation does occur, let the mass of solvent fixed to the particles be w per gm. Then the volume of the hydrodynamic particle in the expression for $\bar{f}$ is related to the volume V^1 of the dry particle by the relation:

$$V = M\left(V_{sp} + \frac{w}{\rho}\right) \qquad \ldots\ldots (2\cdot19)$$

$$\text{or} \qquad V = V^1\left(1 + \frac{w}{\rho\, V_{sp}}\right) \qquad \ldots\ldots (2{\cdot}19^1)$$

where ρ is the density of the solvated layer. Then according to (2·8):

$$\bar{f} = \frac{6\nu}{1{\cdot}612}\,\eta_0 \left(1 + \frac{w}{\rho V_{sp}}\right)^{\frac{1}{3}} (V^1)^{\frac{1}{3}}\,\frac{1}{t(p)}$$

In this expression $\bar{f}$ is known, as is V^1 (from pycnometry). It follows that experiment gives the value of the product:

$$P = \left(1 + \frac{w}{\rho V_{sp}}\right)^{\frac{1}{3}} \frac{1}{t(p)} \qquad \ldots\ldots (2{\cdot}20)$$

If the particle is spherical $t(p) = 1$ and hence the value of either w/ρ or of V is obtained from equation (2·19).

If the particle is ellipsoidal, the problem becomes indeterminate. However, if different values are given to w/ρ, equation (2·20) gives the corresponding value of p, and equation (2·19^1) gives that of V. A new quantity may be introduced; *e.g.* the intrinsic viscosity which involves w/ρ in the equation:

$$[\eta] = \left(V_{sp} + \frac{w}{\rho}\right) \Lambda(p) \qquad \ldots\ldots (2{\cdot}21)$$

The three unknowns V, p and w/ρ may be calculated from the three equations (2·19) or (2·19^1), and (2·20) and (2·21) and the value of M may then be obtained from the density*.

(*c*) All the above analysis is valid in the case where the dispersed particles are ellipsoids of revolution.

It is evident that this is not necessarily the case and it is quite possible that the particle may not have an ellipsoidal shape. In this case the values of f, C and $[\eta]$ can still be defined and measured, but their theoretical expressions are no longer known as functions of the dimensions of the particle.

If the same expressions as above are applied, the three numbers p, V, M, which represent the dimensions and the mass of the fictitious ellipsoid, called the equivalent ellipsoid, can be calculated from these three values as has been shown. But it is clear that these dimensions and this mass can be totally different from those of the actual particle. Besides this there is an ambiguity since it will be possible to define two equivalent ellipsoids, one long and the other flat. Comparison with the actual mass M, supposedly known from other methods, does not clear the ambiguity since

* For discussion of the study of hydration see J. T. EDSALL (General literature).

the observed deviation can be due to the fact that the particle is no longer ellipsoidal.

In particular if, from the measurement of V_{sp} and from the exact mass of the actual particle, the real (dry) volume is calculated $V^1 = V_{sp}\, M$, there is no reason why $V^1 = V$, even if the real particle is not spongelike (lacunar) and is not dissolved.

In these conditions, at least without previous knowledge of the shape of the particle, it is clearly impossible from these methods to obtain other than hypothetical data on the solvated layers. This criticism is of particular value in the study of proteins of small mass, of which mention will be made later.

Instead of defining an equivalent ellipsoid, it is possible to define an equivalent cylinder of revolution by calculating p, V and M from the expressions for f, C and $[\eta]$ valid for such a cylinder. (See appendix).

If it were known in advance that the particle were elongated, then, for example, the actual mass could be compared with the two values calculated for M for the ellipsoidal and the cylindrical shapes. If for the actual particle that of the two shapes which gives the nearest mass to the actual mass is used, then a conclusion can be reached.

In particular, if the particle is considerably elongated (p being some multiple of ten, say,) the expressions for f, C and $[\eta]$ as functions of a and of b are identical. Consequently from these three values, the exact values of a, b and M can be calculated, without knowing whether the particle is an ellipsoid or a cylinder.

On the contrary, if f, C and $[\eta]$ are expressed as functions of p and V, two series of expressions are obtained, given below, and which differ only in the factor which represents the ratio of the volumes of a cylinder and an ellipsoid having the same values of the dimensions a and b.

Ellipsoid of revolution :		*Cylinder of revolution :*	
$f = \dfrac{6\pi}{1{\cdot}612}\ \eta_0 V^{\frac{1}{3}}\ 1/t(p$	(8)	$f = 6\pi\ n_0\ V^{\frac{1}{3}} \cdot 1/t(p)$	. . . . (2·22)
$C = 6\eta_0 V \cdot 1/r(p)$	(9)	$C = 6\eta_0\ V\,\dfrac{2}{3} \cdot 1/r(p)$	. . . . (2.23)
$[\eta] = \dfrac{V}{M}\ A(p)$	(17)	$[\eta] = \dfrac{V}{M}\,\dfrac{2}{3}\ A(p)$	. . . . (2·24)

Meanwhile it is necessary to note that the elimination of the volume V between equations (2·8) and (2·9), or between (2·22) and (2·23) leads to the same expression for p. In this way, from more convenient expressions for the functions of volume and the axial ratio, p can be determined without the necessity of clearing the ambiguity.

When it is certain that the particle is not lacunar, and that its hydration layer is negligible, then V/M is given experimentally by V_{sp}, and by inserting its value in equation (2·17) or (2·24) it is possible to distinguish in principle between the cylindrical case and the ellipsoidal.

Such discussion need be taken no further. It is evident from what has just been said that each particular case must be treated with circumspection, so as to include all possible data provided by other sources (chemical, biological, x-ray crystallography).

It is equally true that any discussion as to precise values found for the dimensions, without prior accumulation of all possible reasons for the acceptance of a shape determined for the particle, is quite useless and there is a risk of its leading to considerable error.

This opinion will be further reinforced when the large uncertainty of the data given by experiment has been indicated—notwithstanding the apparent accuracy of the measurements.

3. OPTICAL METHODS

These methods rely, as has already been stated, on the determination of the perturbation which the dissolved particles produce on the propagation of an electro-magnetic wave in the liquid solvent.

Practically, this is confined to the case where the longest incident wavelengths are in the visible region, the shortest being in the x-ray region.

The case where the particles absorb the incident light will be neglected and consequently it remains to consider how the dissolved particles influence : (*i*) the velocity of propagation of the incident waves; (*ii*) the scattering of these waves in all directions.

The first of these problems, which is that of the influence of the particles on the refractive index of the solution, has not been treated to any extent from the point of view concerned here *i.e.* the determination of the dimensions of particles, and in consequence no guiding principles have been established.

The other problem, on the contrary, has been the object of a considerable amount of work, which has lead to very interesting results.

It follows that this chapter, in spite of the generality of its title, will be devoted entirely to the uses of the methods of scattering of light for the determination of macromolecular dimensions.

The case where the refractive indices of the substance of the particle and the solvent are not nearly equal will not be considered here; it has been treated by MIE[9] for the case of spherical particles. It will be admitted that for biological substances the indices may be practically identical, which leads to a field where a theoretical study of non-spherical particles can be commenced. It is necessary, meanwhile, to remember that the

fundamental simplifications taken *a priori*, will have to be justified in each particular case.

The fundamental hypothesis on which the non-quantitative theory of the scattering of light relies is that the scattering element consists of a small dipole vibrating in phase with the incident ray which, in consequence, radiates in turn electromagnetic waves of frequency equal to the incident frequency.

Hence, the molecule in solution consists of an assembly of dipoles distributed throughout the whole volume which they occupy. Thus there are two cases to be considered :

(*a*) where the dimensions of the assembly are small compared with the incident wavelength and all the dipoles vibrate in phase and behave as one dipole.

(*b*) where the dimensions of the assembly are not small compared with the incident wavelength, and the phenomenon of interference occurs between the waves emitted by the different dipoles.

The energy diffused by the molecule, and consequently by the solution, is not expressed in the two cases by the same equation and, in particular, the shape and dimensions of the particle appear in the second one.

A. THE CASE OF 'SMALL' PARTICLES

The theory shows that the particle may be considered as being small when its dimensions are less than a tenth or a twentieth of the incident wavelength.

In this case the energy scattered by a cc of the solution will be obtained by calculating the energy diffused by the ν particles, each of which behaves as a dipole. If the particles, supposed to be all identical, were

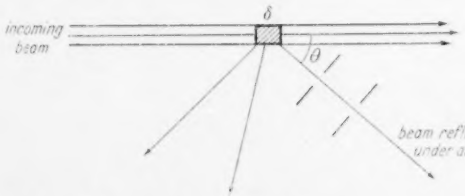


Figure 4.

disposed regularly with regard to each other *e.g.* in a crystal lattice, then interference patterns would be produced and there would be no scattered rays. It is because these particles, undergoing Brownian movement, are distributed at random in the solution that the interference does not completely annul all the scattered intensity, which can be calculated by two different methods provided that the solution is very dilute. The result of the Einstein method* will be given below. This has the advantage

* See General literature at the end.

of being valid for concentrations which are very small, but which are not zero.

Consider a wavefront of ordinary parallel light* of intensity I_0 (Figure 4) to pass through the solution, and consider the luminous energy scattered by an element of volume of the solution, ϕ. It can be shown that the illumination produced by the light scattered by this element of volume ϕ to an element of surface at a distance r from the centre of the volume ϕ is given by the equation :

$$E_\theta = \boldsymbol{R}\,\phi\,I_0\,(1 + \cos^2\theta) \qquad \ldots\ldots (3{\cdot}1)$$

From which we may immediately obtain :

$$\boldsymbol{R}\,\phi = E_{90^\circ}/I_0$$

$\boldsymbol{R}$ is the Rayleigh constant, which theory shows is related to the characteristics of the solution by the equation :

$$Kc/\boldsymbol{R} = (1/M) + 2Bc \qquad \ldots\ldots (3{\cdot}2)$$

where :

$$K = 2\pi^2\,n_0^2\,(dn/dc)^2/N\lambda^4 \qquad \ldots\ldots (3{\cdot}3)$$

c = the concentration of the solution in gm/cc.
n_0 = the refractive index of the pure solvent, n that of the solution.
M = the mass of the dissolved particles.
N = AVOGADRO's number.
B = the second virial coefficient, mentioned later.
λ = the wavelength of the incident ray.

The total energy scattered in all directions by ϕ can also be calculated. This energy is obviously proportional to ϕ and to I_0, the intensity of the incident wavelengths.

This is equal to $I_0\,\phi\,\tau$ where τ is a factor smaller than unity called the turbidity of the solution† which is given by expressions comparable to the above *i.e.*:

$$H\,c/\tau = \frac{1}{M} + 2Bc \qquad \ldots\ldots (3{\cdot}4)$$

with

$$H = 32\,\pi^3\,n_0^2\,(dn/dc)^2/3N\,\lambda^4 = \frac{16K\pi}{3} \qquad \ldots\ldots (3{\cdot}5)$$

* The study of the depolarisation by scattering of the plane polarised light will be omitted in order not to overburden this review.

† It is evident that if I is the intensity of the wavefront after passing through the solution, then :

$$I = I_0\,e^{-\tau\lambda}$$

Thus τ is also the coefficient of apparent absorption of the solution.

This shows that the masses of the particles contained by a solution may be determined from the study of the light scattered by the solution.

Hence it suffices to measure, for example, the Rayleigh constant for the same solution at different concentrations. Plotting the different values of $Kc/\boldsymbol{R}$ as ordinates and those of c as abscissae will give, at least in the region of small values of c, for which the theory is valid, a straight line, of which the slope gives B and the intercept on the vertical axis gives $1/M$.

This method, is very elegant but provokes certain criticisms.

Firstly, it is difficult to measure the Rayleigh constant directly. In general, such absolute measurements are only made in the study of scattering of light by pure liquids, which scatter much less than the solutions. For the application to the determination of the masses of colloidal particles it is usually sufficient to measure the quantity E_{90}, which is proportional to the Rayleigh constant and the proportionality factor can be determined by standardising with a liquid, of which the scattering has been determined absolutely, and for which the ratio E_{90}/I_0 is known. It so happens that actually there is considerable uncertainty concerning the values of the standards used, which undoubtedly constitutes a considerable difficulty and limits the application of the method. (See Section III).

Finally, other criticisms of a theoretical nature appear, which may be stressed : one relates particularly to the coefficient of the internal field which makes the numerical coefficient found in equation (3·3) uncertain; the other relates to the effect which the disturbance has on the adsorption and thus on the scattering of the solvent itself.

Even if it is certain that the method gives the order of magnitude of the masses of the dissolved particles correctly, it is still difficult to estimate its accuracy.

B. THE CASE OF LARGE PARTICLES

In this case each particle can be represented by a collection of dipoles which, because of their relative positions, do not vibrate in phase. The waves which they emit interfere and theory shows that the energy scattered by a small volume of the solution is distributed dissymmetrically in space, being larger in the direction of propagation of the incident wavefront (θ small) than in the opposite direction (θ near to π).

In this case, equation (3·1) must be replaced by the more general expression :

$$E_\theta = \boldsymbol{R}\,\phi\, I_0\,(1 + \cos^2\theta)\,P(\theta) \qquad \ldots\ldots (3\cdot6)$$

The factor $P(\theta)$ can be calculated theoretically if the geometrical shape of the particle is known and if the index of refraction of the latter is very nearly equal to that of the solvent.

Hence :

(*a*) for a sphere : $P(\theta) = [3x^{-3}(\sin x - x \cos x)]^2$ . . . . (3·7a)

where $x = \beta sa$

(*b*) for a rod of revolution :

$$P(\theta) = \frac{1}{x}\int_0^{2x} \frac{\sin w}{w} \cdot dw - \left(\frac{\sin x}{x}\right)^2 \qquad \ldots . (3{\cdot}7b)$$

where $x = \beta sa$

To this we can add an expression which, whilst we are not concerned with this type of particle here, relates to a Gaussian macromolecular chain :

$$P(\theta) = 2x^{-2}[e^{-x} - (1 - x)], \text{ where } x = (\beta s)^2 \bar{l}^2/6 \quad \ldots . (3{\cdot}7c)$$

In these expressions a represents the radius of the sphere, or the half length of the rod, $\bar{l}^2$ the mean square of the distance between the ends of the chain, s is equal to $2 \sin \frac{\theta}{2}$ and $\beta = 2\pi/\lambda^1$ where λ^1 is the wavelength of the light in the solution.

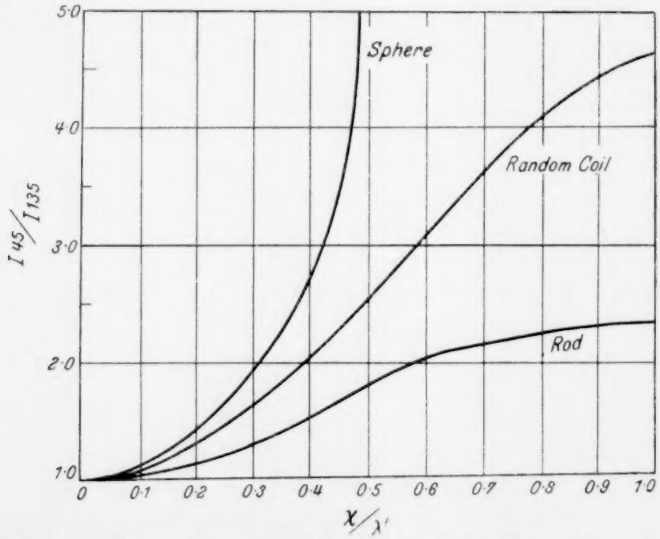


Figure 5. Dissymmetry of diffuse light as function of x/λ' where $\lambda' = \lambda z v$ (after OSTER[19]).

If the shape of the particle in solution is known the comparison between the experimental graph of the distribution of the scattered energy and the theoretical graph would give a or $\bar{l}^2$.

The methods of comparison used in practice will not be dwelt on here*, and it will only be indicated that one of these consists of comparing

* See page 277.

the scattered intensity in two symmetrical directions, for example for $\theta = 45°$ and $\theta = 135°$.

The ratio $I_{45°}/I_{135°}$ is then equal to $P\,(45°)/\,P\,(135°)$ and for a given geometric shape, it is a known function of x/λ^1 where x is either a or $(\bar{l}^2)^{\frac{1}{2}}$ corresponding to the case in question. These functions are shown in Figure 5.

Thus it is clear that measurement of $I_{45°}/I_{135°}$ leads directly to the value of x/λ^1, and thence to that of x.

Finally when the dimensions of particles of a given shape have been measured in this way the numerical value of $P\,(\theta)$ is known and consequently from (3·6) it is possible to calculate the value of $\boldsymbol{R}$ from that of E_θ. It is then possible to determine $1/M$ and B as indicated above in (3·2) and (3·3) for small particles.

Hence when the particle is large it only remains to make a correction for dissymmetry arising from the dissymmetry factor $P\,(\theta)$.

C. SCATTERING OF X-RAYS*

It has become evident that the effect of dissymmetry, on which the measurement of the dimensions of the particles is based, appears when they are longer than about ten times the wavelength of the incident light. If the method was limited to the use of visible light it would only be conveniently applicable to particles of dimensions greater than 500 Å.

The use of x-rays lowers this limit considerably.

The study of the dimensions of particles by the scattering of x-rays relies on the principles which have just been enunciated and which are valid whatever may be the frequency employed. However in practice there are some differences. Firstly it can be shown that the dissymmetry is considerably accentuated so that the ratio of the dimensions of the particle to the wavelength of the incident ray is increased.

Now, in general, the dissymmetry will be considerable for x-rays, which means that the scattered energy will be localised in the region where θ is very small.

It is for this reason that the method is called 'the method of scattering of x-rays at small angles'.

On the other hand, since θ is small the magnitudes of x in the expression for $P\,(\theta)$ are small and it is possible to replace the exact expressions by a limited expression.

Hence, whatever the shape of the particle:

$$E\,(\theta) = A \exp\,(\,-\,4\pi^2\,R_0^2\;\theta^2/3\lambda^2) \qquad \ldots\,. (3{\cdot}8)$$

In this expression R_0 is the radius of gyration of the particle which

* See General literature.

corresponds to a general definition which need not be explained further; it leads to the following expressions :

for an ellipsoid $$R_0^2 = \frac{2a^3 + b^3}{5a}$$

for a sphere $$R_0^2 = \frac{6\,a^2}{10}$$

for a rod $$R_0^2 = \frac{a^2}{3}$$

for a Gaussian particle $$R_0^2 = \frac{\bar{l}^2}{3}$$

The value of R_0 is obtained experimentally in the determination of $\bar{I}(\theta)$ by photography or by counting photons. The slope of the graph giving Lim $\bar{I}(\theta)$ as a function of θ gives R_0^2 from (3·8).

Practically therefore, the use of x-rays allows the determination of the radius of gyration of the dissolved particles, and also, if their shape is known *a priori*, the calculation of their dimensions.

In conclusion, the study of the scattering of light by a solution of large particles permits calculation of their masses and dimensions according to the conditions already given.

For this reason, this method is of considerable interest. Nevertheless, it is necessary to recall that when put into operation, it encounters certain difficulties. Firstly, as for hydrodynamic methods, the theoretical expressions are only valid for infinitely small concentrations. It is therefore necessary, for a given type of solution, to make determinations at different concentrations and to extrapolate the results obtained to zero concentration. Several practical complications follow. Several methods of extrapolation have been proposed to obviate the principle difficulties[10].

Since the dissymmetry is the more important as the size of the particles increases, it follows that the existence of dust, even to a small extent, involves considerable errors. It is therefore absolutely necessary to clarify the solutions completely, which is not always very easy. The centrifuge provides perhaps the most certain means of doing this.

Finally, in the case of large particles, the theory can be submitted to the same criticisms as apply in the case of small particles, and even to some additional ones.

Thus, for example, HORN, BENOIT and OSTER[11], assuming another hypothesis than that of DEBYE for the isotropy of the elementary oscillators which constitute the large molecule, found, in the case of a rod, another expression for the dissymmetry factor. They assumed that the dipoles had

two principal polarisabilities, the one being α parallel to the rod, and the other, β, in the direction perpendicular to the rod. Then, the dissymmetry of the scattered light depends on the optical anisotropy $\delta = (\alpha - \beta)/(\alpha + 2\beta)$ of the rod. The diagram of Figure 6 gives the new values of I_{45}/I_{135},

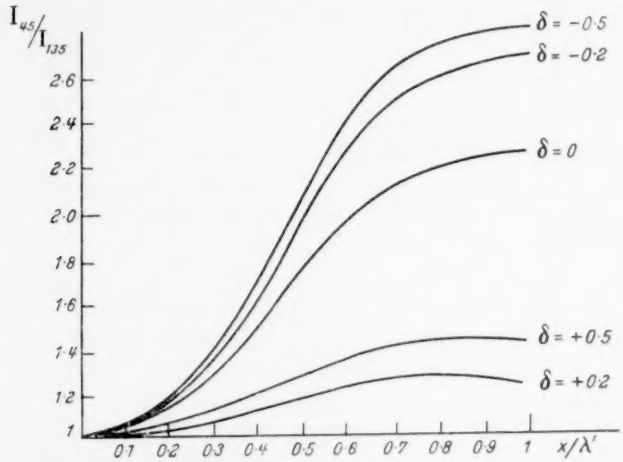


Figure 6. Dissymmetry of diffuse light as function of α/λ' in case of optical anisotropy (after HORN, BENOIT, OSTER[11]).

as a function of x/λ^1 for different values of δ. Obviously, for $\delta = 0$ the curve is that given by Debye's theory.

According to DEBYE the maximum value of the dissymmetry is equal to approximately 2·3, so that, taking anisotropy into consideration, it can vary between 3 (for $\delta = -0{\cdot}5$) and 1·3 (for $\delta = 1$).

Thus, according to the value of δ, interpretation of the measurements of dissymmetry will therefore give a different value for the length of the rod.

Before ending this section it is necessary, as already stated, to discuss the significance of the dimensions measured by optical diffusion, as in the case of the hydrodynamic methods.

Here there are no subtle distinctions to be drawn. The diffusion which is measured is produced by the elements of the dissolved matter. Therefore the dimensions obtained are those of the space occupied by the matter of the dissolved particle. These dimensions are not necessarily those of the 'dry' particle. For example, the particle may be inflated, due to the penetration of the solvent into its interior. It is then the volume of the inflated particle which causes scattering of the light.

On the contrary, in principle, the superficially adsorbed solvent plays no part in the scattering[12]. The scattering of the light can not therefore by itself show the arrangement of the adsorbed films.

However, if the adsorbed solvent has a different index of refraction from that of the free solvent the adsorbed film will scatter light too and, in consequence, the dimensions measured will no longer be those of the dissolved particle. Further, the adsorbed solvent scatters differently from the free solvent so that a correction must also be made on this account.

These few remarks show that the method of light scattering, because of these possibilities, is still far from giving unambiguous results. Consequently, in order to appreciate the value of the latter, it is indispensable to have recourse to data obtained from entirely different considerations.

THE INFLUENCE OF POLYDISPERSITY

When the solution contains a mixture of particles of different shapes and dimensions, it is obvious that the methods described above are no longer applicable.

If the experimenter, unaware of the heterogenity of the solution being studied, has submitted it meanwhile to the analysis just described, the results obtained will indicate a type of 'mean' particle, of which the characteristics will all too often not be of any use to define the mixture. It can even happen that in certain conditions, it will lead to obvious absurdities.

It is necessary therefore, from this point of view, as well as from a much more general point of view, to be able to recognise in the first place whether or not a solution is monodisperse. After this the determination of the law of distribution for different types of mixtures will be discussed (See I, p. 240), a problem which is far from being solved in a satisfactory manner.

A. EXPERIMENTAL CHARACTERISTICS OF A MONODISPERSE SOLUTION. THE USE OF MEAN MEASUREMENTS

Consider, as has already been indicated in Chapter I, a solution containing a mixture of particles and let $\nu_i/\nu = \nu_i'$ be the percentage of particles of type i.

For simplicity, and also because it corresponds to the most important case, only the mass will be considered, and the operations which permit the latter to be determined when applied to a monodisperse solution will be applied to the polydisperse solution.

Only the determinations of the osmotic pressure and the Rayleigh constant will be examined here.

Osmotic pressure. The osmotic pressure, π, of the mixture, is the sum of the osmotic pressures of its constituents and at sufficiently small concentrations, according to VAN T'HOFF :

$$\pi = \Sigma_i \pi_i = RT \, \Sigma_i \, c_i/M_i$$

where R is the gas constant for a perfect gas.

If it is supposed that the solution is monodisperse, and if c is the total concentration of the mixture in gm/cc the mass attributable to the mean molecule is such that :

$$\pi = RT \cdot c/\overline{M}_n$$

from which : $$\overline{M}_n = c/\Sigma_i\, c_i/M \qquad \ldots\ (4{\cdot}1)$$

or again : $$\overline{M}_n = \Sigma_i\, \nu'_i\, M_i$$

where $\overline{M}_n$ is the mean numerical mass.

According to the definition of the Rayleigh constant, the constant measured will be the sum of the constants $\boldsymbol{R}_i$ corresponding to each type, and according to equation (3·2), where the term Bc for the concentration is neglected :

$$\boldsymbol{R} = \Sigma_i\, \boldsymbol{R}_i = \Sigma_i\, K_i\, c_i\, M_i \qquad \ldots\ (4{\cdot}2)$$

If it is supposed that all the types have the same index, all the values of K_i are equal, and in treating the solution as though it were monodisperse and contained particles of identical masses equal to $\overline{M}_p$, according to equation (4·2) we obtain :

$$\overline{M}_p = \frac{1}{c}\, \Sigma_i\, c_i\, M_i \qquad \ldots\ (4{\cdot}3)$$

or again

$$\overline{M}_p = \Sigma_i\, (\nu'_i\, M_i^2)/\Sigma_i\, (\nu'_i\, M_i)$$

where $\overline{M}_p$ is the mean gravimetric mass.

It is clear from these two examples that, in general, the mean numerical and gravimetric masses are different, except when the solution only contains one type *i.e.* if it is monodisperse. The comparison between the masses obtained from osmotic pressure and Rayleigh diffusion for the same solution will show whether or not the solution is polydisperse. If the experiments are carried out carefully, the variation between the two numbers can in some measure serve to characterise the degree of polydispersity of the solution.

B. OTHER METHODS. DETERMINATION OF THE DISTRIBUTION LAW

The other methods* which can be used to reveal the polydispersity of a solution all rely on the same principle : they consist in observing whether a phenomenon exhibited by the solution in the experiment conforms, in its behaviour, with that shown theoretically in the case of a monodisperse solution. This comparison is not always easy, for the theoretical behaviour of a solution is only well known when the concentration of the solution

* Methods relying on the difference in solubility between the different types and methods relying on all other principles than those reviewed in the preceding pages have been ignored here.

is very small : for concentrations used experimentally it can happen that the effect of concentration can invalidate all comparison. It is only in rare cases that these methods can be pushed to the extent necessary for a quantitative determination of the distribution curve for the molecular species, even when the latter are in very small number (pauci-dispersity). In simple cases, in fact, it is known that the use of sedimentation, Brownian diffusion, birefringence of flow, electrophoresis *etc.* will show the existence of dispersity and also allow the distribution of the species contained in the solution to be estimated.

If, on the contrary, the distribution of the species is practically continuous, only methods such as those relying on the Brownian diffusion of the mixture are useful.

The latter can be classified in two categories : in the first* the curve of the concentration $c\ (x, t)$ is determined at different times in the diffusion cell, or more usually, the curve of dc/dx. If the medium is polydisperse, the distribution functions $\nu_i\ (\Delta_i)$ are determined such that the sum of concentration ν_i calculated theoretically as functions of x and of t represent the experimental curve $c\ (x, t)$[13].

Obviously there is an infinity of distributions $\nu_i\ (\Delta_i)$ satisfying these conditions so that the method is far from perfect. This can be avoided meanwhile by examining a second molecular characteristic, ρ_i, whose relation to Δ_i is known.

The second category consists of methods comparable to the former, but in which the rate of diffusion plays a fundamental rôle.

1. *Translational diffusion*—Suppose for example, that the value of $\nu\ (x, t)$ is determined as a function of time†, or more exactly, that of $\dfrac{\delta\nu\ (x, t)}{\delta\ t}$ for a point determined by its depth below the initial surface of separation between the solution and the solvent. If γ_i is the initial concentration of the solution for a monodisperse medium of the type defined by Δ_i, then according to (2·2) :

$$\frac{\delta\nu_i}{\delta t} = \frac{1}{4}\,\gamma_i\,\frac{a\,t^{-3/2}}{\sqrt{\pi\Delta_j}}\,e^{-a^2/4\,\Delta_i t}$$

whence :

$$4\,\sqrt{\pi}\,\frac{t^{3/2}}{a}\cdot\frac{\delta\nu_i}{\delta\,t} = \gamma_i\,\Delta_i^{-\frac{1}{2}}\,e^{-a^2/4\,\Delta_i t}$$

* The Brownian diffusion can be obtained at the same time as the sedimentation. Hence the first category supports the analysis of polydispersity by the ultracentrifuge method. In our opinion these methods are inconvenienced by the necessity for too high concentrations for the effect of polydispersity to be completely separated from that of concentration.

† In practice neither ν nor γ, but the index of refraction of the solution at these concentrations is measured. It is necessary therefore to be sure that the indices are proportional to the concentrations, which is generally the case with the small concentrations used.

If the medium is polydisperse then clearly :

$$4\sqrt{\pi}\,\frac{t^{3/2}}{a}\cdot\frac{\delta v}{\delta t} = \Sigma_i\,(\gamma_i\,\Delta_i^{-\frac{1}{2}}\,e^{-a^2/4\Delta_i t}) = y(t)$$

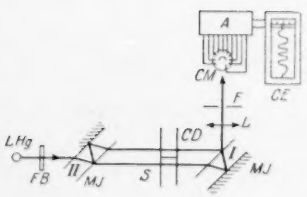
The magnitude of the first factor which appears in this equation can be obtained from experiment. As for the second, it can be written explicitly :

$$\gamma_1\Delta_1^{-\frac{1}{2}}\,e^{-a^2/4\Delta_1 t}\left[1+\frac{\gamma_2}{\gamma_1}\left(\frac{\Delta_2}{\Delta_1}\right)^{\frac{1}{2}}e^{-a^2/4t\left[\frac{1}{\Delta_2}-\frac{1}{\Delta_1}\right]}+\cdots\right]$$

If it is supposed that Δ_1 represents the greatest value of Δ_i, for very small times this expression reduces to :

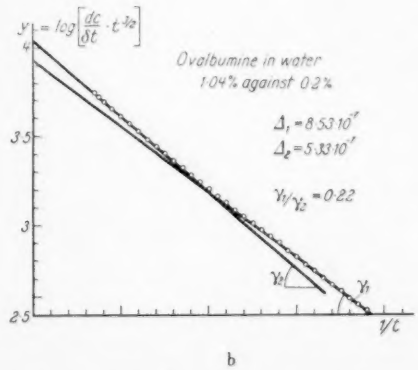
$$\gamma_1\;\Delta_1^{-\frac{1}{2}}\,e^{-a^2/4\Delta_i t} = y_1(t)$$

The beginning of $y_1\,(t)$ of the curve $y\,(t)$ thus gives the value of Δ_1 as well as that of γ_1. Having Δ_1 and γ_1, the curve $y_1\,(t)$ is subtracted from



a

Figure 7a
Apparatus for determining diffusion.



b

Figure 7b
Measurements with an ovalbumen solution showing presence of two macromolecular species.

the total curve plotted $y\,(t)$ and the operation is repeated. This is the principle of the method elaborated by SCHEIBLING[14] (Figure 7).

2. *Rotational diffusion*—BENOIT's method for the determination of D (see p. 250) can be used according to the preceding principles. In the case of a polydisperse medium experiment gives the curve :

$$y(t) = \Sigma_i\;\gamma_i\;e^{-6Dit}$$

where γ^i is the concentration in the mixture of the species for which the

constant of rotational diffusion is D_i. Expanding the expression containing the smallest of the values for D_i (the constant of diffusion) *i.e.* D_1, in accordance with all the above,

$$y(t) = \gamma_1 e^{-6D_1 t} [1 + \epsilon_2 + \epsilon_3 + \ldots .]$$

where ϵ_1, ϵ_2, are terms which tend to zero as t tends to infinity. The curve y (t) for very large values of t thus gives D_1 and γ_1. Hence the quantity $\gamma_1 e^{-6D_1 t}$ may be obtained and subtracted from y (t). The new curve obtained gives D_2 and so on (Figure 8). These methods can only give an overall estimation of the distribution function. In order that they may be used rigorously it is therefore necessary that the curve y (t) should

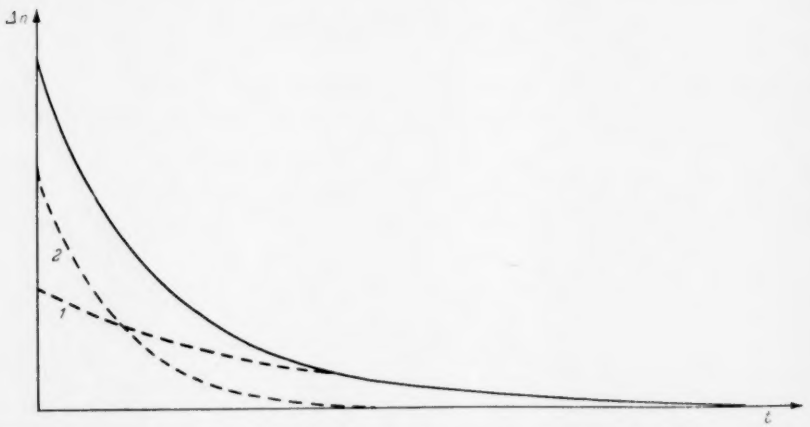


Figure 8. Complete experimental curve of disappearance of electric birefringence as function of time for a solution of sodium thymo nucleate (BENOIT[33]). It has, for large values of t, an exponential form, corresponding to the diffusion constant D. This corresponds to curve 1, which when subtracted from the experimental curve, leaves curve 2, also practically exponential, with constant D_2. In this case the analysis indicates only two components.

correspond to zero concentration, as the algebraic expression used is only correct in that case. Further it is easily seen that the errors accumulate in the process of subtraction.

Such as they are, however, these methods give results which are as satisfactory as those obtained by ultracentrifuge methods, and with less imposing apparatus.

3. Finally, in the case where it is possible to photograph the particles by means of the electron microscope, it is clear that the distribution function may be deduced from the examination of a large number of images without any further difficulties. The next chapter contains some examples of the use of this singularly direct method.

III. EXPERIMENTAL EXAMPLES

The determination of the dimensions of colloidal particles according to the methods just given relies on the use of numerical expressions relating to the magnitudes to be determined (volume, axial ratio of an ellipsoid of revolution, mass *etc.*) to the experimentally measurable magnitudes (coefficient of hydrodynamic friction, viscosity, intensity of light scattering *etc.*).

These expressions have been established from theoretical considerations which themselves often rely on simplifying hypotheses.

In particular it has been supposed that the particles are sufficiently large, taking Brownian movement into account, for the solvent to be considered as a continuous fluid. It is necessary by all accounts to define the expression "sufficiently large" more exactly in order to know whether the application of theoretical formulae is possible.

It is for this reason that it is necessary to verify the theoretical laws experimentally and to find at what dimensions the particle becomes too small for the theories to be valid. One could hope to be able, thanks to sufficiently refined techniques, to establish empirically the exact fundamental laws to which theory only gives an approximation.

The first part of this chapter will be limited to the examination of some experimental verifications. The second will contain some examples of general methods for the study of particular cases.

1. EXAMPLES OF EXPERIMENTAL VERIFICATIONS

There are two ways in which the verification of the theoretical laws could be undertaken.

The first, which leads to a complete verification, supposes that the shape and dimensions of the particle in solution and also the thickness of the solvated film which it adsorbs are known in advance. In this case, comparison between experimental results and those obtained by calculation from the known dimensions indicates whether or not the expressions are the correct ones.

The second is of a less absolute character than the first. It consists of calculating, for example, the volume and the axial ratio of the ellipsoid by the application of two theoretical expressions and seeing whether, on putting the values obtained in a third expression containing these two parameters, the latter is verified.

The work of verification is usually based on the use of these two principles and is done in an empirical manner. Thus the dimensions of the particle are determined by means of the electron microscope and then it is found whether the values calculated from these dimensions are in agreement with the values which have been measured.

It has already been stated that it is not certain that the electron photograph gives the dimensions and shape of the particle in solution. This type of verification therefore supposes the physical-chemical properties of the particle which has been chosen for the model to be known in advance.

It is for these reasons that particles are used which are already known to have a negligible solvated layer, and whose structure is not lacunar, and where in consequence, the identity of the electron photograph and of the particle in solution is as certain as possible. In these conditions, if the experimental agreement between the calculated and observed values is compatible with experimental error, the validity of the law under examination may then be assumed.

A. USE OF SOLUTIONS OF THE TOBACCO MOSAIC VIRUS

The particles of the tobacco mosaic virus appear as rods of thickness, determined by X-rays[16], equal to 152 Å and of length, determined by the electron microscope, in the order of 2,700 to 2,800 Å. In fact, although the thickness of the rods is appreciably constant, the same is not true of their lengths which depend on the care taken with their preparation. Some authorities claim that all the rods are of the same length when certain precautions are taken.

It would seem therefore, that the particles form an ideal choice: the nucleoprotein of which they consist is a dense material and different estimations seem to show that the hydration film which envelopes the rod is only of the order of a few angstroms[17] *i.e.* it may be neglected without appreciable error in comparison with the mediocre accuracy of the experiments.

It is quite correct therefore to assume *a priori* that electron photography gives the dimensions of the particles in solution.

Such suspensions have been used to verify the validity of the theoretical expressions for the intrinsic viscosity, for the rotational translational diffusion constants, and for the dissymmetry of the scattered light.

(*a*) *Viscosity*

Solutions of the virus which the electron microscope has shown to be almost completely monodisperse have already been dealt with.[18]

In the expression for the viscosity (2·17 or 2·24) the value of V/M is determined by pycnometry, and this gives the correct value here since the substance of the particle is not lacunar and the hydration film is negligible. V/M has been found to be 0·73. The value of $A\,(p)$ or of p may be obtained from that for $[\eta]$. The following table gives the values calculated for p in the cases of the ellipsoid and of the cylinder of revolution. If the calculated values of p seem nearest to the measured values obtained for the ellipsoid model, it should be noted that at least the cylinder is in the greatest conformity with the photographs.

		p (photography)	p (ellip) calc.	p (cyl) calc.
LAUFFER	285	$18 < p < 19$	20·3	25·9
OSTER, DOTY, ZIMM	28	$18 < p < 19$	20·0	25·

(*b*) *Translational diffusion—sedimentation*

Of the different determinations which have been made, those of Lauffer will be quoted, which give for the translational diffusion constant

$$\Delta = 5{\cdot}3.\ 10^{-8} \pm 8 \text{ per cent}$$

At this temperature (20°):

$$kT = 4{\cdot}04.10^{-14}$$

whence:

$$\bar{f} = kT/\Delta = 7{\cdot}62.\ 10^{-7}$$

The value of $\bar{f}$ calculated from formula (2·2) in which the following dimensions of the rod were substituted gave $\bar{f} = 6{\cdot}4\ .\ 10^{-7}$,

$b = 76.10^{-8}$ cm $\qquad$ $p = 18{\cdot}4$

whence

$a = 1400.10^{-8}$ cm $\qquad$ $V = 33{\cdot}8.10^{-18}$ (ellipsoid)

and

$V = 51.10^{-18}$ (cylinder)

Taking for V the value obtained by electron measurements and for V/M that of the specific volume measurements:

M (ellipse) $= 46.10^{-18}$ $\qquad$ M (cyl.) $= 69.10^{-18}$

With the measured value for $\bar{f}$ obtained from Δ, S may be calculated as follows:

$$S = M\,(1 - V_{sp}\rho)/\bar{f} = 1.8.10^{-11}$$

The measured value is equal to $1{\cdot}85 \times 10^{-11}$.

(*c*) *Rotational diffusion constant about an equatorial diameter*

Two types of verification have been made, one by means of the birefringence of flow, and the other by means of the Kerr effect.

In these experiments the greatest account has been taken of the polydispersity of the solutions employed in determining the statistical distribution of the lengths of the rods from the electron microscope photographs.

Birefringence of flow—DONNET[19], first using the theory that if the particles are long rods, as in the present case, the value of the rotational diffusion constant measured from the initial slope of the graph of the angle ϕ as a function of the velocity gradient (see Section II, p. 251) is:

$$1/D = [\Sigma_i\ (\nu_i^1/D_i^2)/\Sigma_i\ (\nu_1/D_i)]$$

Using equation (2·9) it is possible to calculate the value of D_i for each type of particle as in the case of D given by the above equation.

Hence it is found that $D = 300 \pm 10\%$ c.g.s.
The measured value is $D = 280 \pm 10\%$ c.g.s.

Kerr effect—As in DONNET's experiments, a preliminary investigation was made by means of the electron microscope of the distribution of the lengths of the rods in the solution used.

It has been indicated that BENOIT's method permitted measurement of $\Sigma \nu_i' e^{-D_i t}$. In fact what is really measured is the variation of the birefringence of the solution as a function of time.

For a polydisperse medium consisting of rods of sufficient length (as in the present case), the latter is given by the expression :

$$\frac{\delta n}{\delta n_0} = \frac{\Sigma_i \nu'_i a_i^2 e^{-6D_i t}}{\Sigma_i \nu'_i a_i^2} \qquad \ldots\ldots (5{\cdot}1)$$

where δn_0 is the initial birefringence of the solution and a_i is the half length of the rods of type i.

On the other hand, from the expression for the diffusion constant D for a long rod (see appendix) the ratio may be written :

$$D_i/D_0 = (a_0/a_i)^2 \qquad \ldots\ldots (5{\cdot}2)$$

Substituting in equation (1) the value of D_i obtained from equation (5·2) gives :

$$\frac{\delta n}{\delta n_o} = \frac{\Sigma_i \nu_i a_i^2 e^{-6D_o(a_o/a_i)^2 t}}{\Sigma_i \nu_i a_i^2} \qquad \ldots\ldots (5{\cdot}3)$$

Examination of the preparation with the electron microscope gives ν_i and a_i, and experiment gives $\delta n/\delta n_0$ as a function of time, therefore D_0 may be calculated from equation (5·3) for a particle of known length, $2a_0$.

The standard value has been taken as :

$$a_0 = 1400\text{Å}.$$

Hence from (5·3) :

$$D_0 = 550 \text{ c.g.s.} \pm 10\%$$

The value calculated from (2·9) is :

$$D_0 = 540 \text{ c.g.s.}$$

(d) Dissymmetry of the scattered light

With the same preparation as that which they used for checking the theoretical expression for the intrinsic viscosity OSTER, DOTY and ZIMM[18] have attempted to verify the validity of the theory of light scattering in the case of large particles.

They used the dissymmetry method *i.e.* they measured the ratio $I_{45°}/I_{135°}$ and worked according to the methods described in the preceding chapter

They found that at zero concentration the ratio $I_{45°}/I_{135°}$ was equal to 1·94 and is the same for solutions in water whether distilled or not.

Using the value they obtained the result:

$$2a = 2700\text{Å}.$$

With the apparatus being standardised with pure benzene, the corresponding mass was:

$$M = 66 \cdot 10^{-18}$$

This value for the mass of the particle, is not influenced by its shape, as in the case of osmotic measurements. Following on this DOTY and STEINER[10] have made measurements by the method of extrapolation. It is noteworthy that the mass calculated from the scattering of light seems to depend on the choice of the angles θ and θ plus 90° in which the intensities of scattering are measured and from which the ratio is obtained. It is difficult to affirm that this variation is due to the theoretical expression $P(\theta)$ not being correct. It is possible that the polydispersity of the medium may be at least partly responsible for this anomaly. In the meantime the uncertainty may be avoided in the following way.

The value of E_θ in equation (3·6) is measured for decreasing values of θ and the value of E_0 is determined by extrapolation to $\theta = 0$. Hence in these conditions, according to equation (3·6) $E_0 = \boldsymbol{R}\,\phi\,\bar{I} \times 2$ for $P(0) = 1$. If the apparatus has been standardised it is possible to calculate $\boldsymbol{R}$ as a function of E_0 and from this to determine M. This method has been made possible by ZIMM who uses a double extrapolation diagram which is a function of c and of θ.

With this method and the same standardisation for their apparatus DOTY and STEINER give the following values:

$$2a = 2550 \pm 100 \text{ Å}.$$
$$M = 83 \cdot 10^{-18} \pm 2\%$$

(*e*) *Conclusion*

From the data available a satisfactory and coherent estimate may be obtained for the dimensions. The hydrodynamic and optical methods for p (or $2a$) give numbers which do not vary more than 10 per cent, and are in agreement with those derived from the electron microscope photographs.

The general formulae which have been applied have therefore been verified within the limits of experimental error for particles whose dimensions are greater than or equal to those of the rods of the tobacco mosaic virus.

There is, on the other hand, a considerable deviation between the masses measured. However, this deviation is less if a cylindrical shape is attributed to the particle, which seems the most likely according to the photographs. This illustrates a remark on page 274. Even if it is admitted

that the particle is cylindrical, the deviations can be attributed to the polydispersity of the sample.

But it is also necessary to point out, returning to another question already raised in the preceding paragraph, that the standards of turbidity possess no absolute certainty. For this reason, DOTY and STEINER used pure benzene as their standard, for which they gave a Rayleigh constant of $48{\cdot}2.10^{-6}$ for $\lambda = 4370$Å and $16{\cdot}4.10^{-6}$ for $\lambda = 5460$ Å whilst CABANNES gave different numbers. Again, the recent measurements of VAUCOULEURS gave[21] $31{\cdot}8.10^{-6}$ for $\lambda = 4350$Å and $11{\cdot}8.10^{-6}$ for $\lambda = 5440$ Å. If these values are taken as standards, the mass measured by DOTY and STEINER reduces to 55.10^{-6}.

B. OTHER EXPERIMENTS

Further experiments of the same type have been tried with other types of particles, but, in general, they do not lend themselves to verification as well as the tobacco mosaic virus.
Some examples follow.

(a) Sols of $V_2 O_5$

The experiments were made on two types of solutions containing particles whose dimensions determined with the electron microscope, were as follows :

Sol I	*Sol II*
$2a = 1500 \pm 200$Å	$2a = 2300 \pm 200$ Å
$2b_1 = 100 \pm 25$ Å	$2b_1 = 100 \pm 25$Å
$2b_2 = 50 \pm 25$Å	$2b_2 = 50 \pm 25$ Å

The existence of two transverse dimensions, b_1 and b_2, shows that the particles resemble rectangular sectioned parallelepipeds.

The experimental error of the dimensions is too large for there to be any hope of verifying the theoretical laws with sufficient precision, but it has been found meanwhile that the results which they give are of the order of magnitude expected.

On the contrary[23] the ratio of the intrinsic viscosities of the two solutions is equal to $A(p_1)/A(p_2)$ and involves neither the value of V/M nor the absolute dimensions of the particles.

If the latter are compared to cylinders of revolution of diameter b_1 plus b_2, $p_2 = 20$ for solution I and $p2 = 31$ for solution II.

The ratio of intrinsic viscosities is :

$$[\eta]_2/[\eta]_1 = 92{\cdot}4/42 = 2{\cdot}2$$

according to the table of values for $A(p)$ it is found that

$$[\eta]_2/[\eta]_1 = A(31)/A(20) = 2{\cdot}0$$

(*b*) *Sols of carbon black*

The sols[24] are prepared from carbon black by oxidation of the surface of the particles with sodium hypochlorite. Examination of such a solution with the electron microscope shows that the particles are spherical and that the statistical distribution of their radii corresponds to the diagram

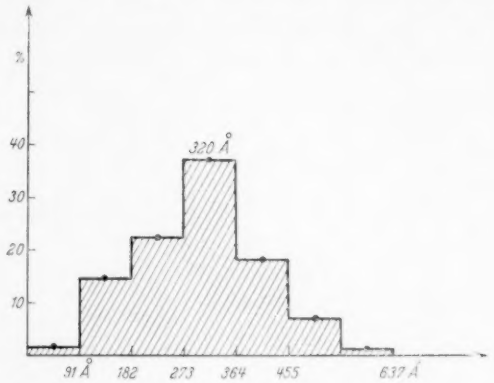


Figure 9. Distribution of diameters of spheres of carbon black, used by DONNET and SADRON[24]. (From electron microscope photographs).

below (Figure 9). For these solutions, only measurements of the Brownian translational diffusion have been made here.

The influence of polydispersity can be introduced more rigorously in this particular case, since it can be shown that, in the method of measurement employed, the mean diffusion constant measured is related to the diffusion constant Δ_i of the different types contained in the solution by the relation :

$$\frac{1}{\Delta} = (\Sigma_i \nu_i / \Delta_i^{\frac{1}{2}})^2$$

The value calculated for Δ from electronic measurements, on the supposition that the value of Δ_i is given by equation (2·8), is :

$$\Delta_{\text{calc.}} = 1{\cdot}4.10^{-7} \pm 10\%$$

The observed value is :

$$\Delta_{\text{obs.}} = 1{\cdot}5.10^{-7} \pm 10\%$$

(*c*) *Study of the Haricot mosaic virus*

This method has been chosen because it illustrates a difficulty of the electron microscope method.

The particles of the Haricot mosaic virus have been photographed with the electron microscope by PRICE, WILLIAM and WYCKOFF[25] and appear as spheres of radius 125 Å. In addition LEONARD, ANDEREGG, KOESBERG, SCHULMAN and BEEMAN[26] have found a radius of 130 Å.

On the other hand, the latter have studied the scattering of these solutions with x-rays at small angles, deriving a value for the radius : $R = 149 \pm 3.3$ Å.

In addition MULLER and PRICE[27] have studied the same virus by hydrodynamic methods. For the Brownian constant of diffusion they give the value $\Delta = 1{\cdot}38.10^{-7}$.

If it is assumed that the particle is a sphere, equation (2·8) for Δ gives $R = 153$Å.

Further the partial specific volume was found to be equal to 0·69 and the sedimentation constant was $S = 115.10^{-1}{}_3$

From the equations $S = M\,[1 - V_{sp}\,\rho]\;\Delta/kT$, $M = 10{\cdot}8.10^{-18}$ is obtained.

Knowing M and knowing $[\eta]$ which has been found equal to 4·40, the value of V may be obtained from the Einstein equation :

$$V = [\eta]\,M/2{\cdot}5 = 10.10^{-18} \text{ cc}$$

The corresponding radius is $R = 165$ Å.

Finally the volume of the dry particle will be $V^1 = V_{sp} \times M$, which is here equal to $2.45.10^{-18}$. The corresponding radius is : $R^1 = 121$ Å.

We see then that

1. The values for the radius obtained by hydrodynamic methods are mutually compatible. In fact their deviations are less than 10 per cent, and are perfectly explained as experimental errors.

2. These values are compatible with the determinations obtained by scattering of x-rays.

3. They are greater by at least 20 per cent than those determined by the electron microscope; this deviation can only with difficulty be attributable to experimental errors (although that is not entirely excluded).

Hence the following conclusions become apparent :

For spheres of the Haricot mosaic virus, the STOKES and EINSTEIN laws appear to remain valid and they are consistent with the law of scattering of x-rays at small angles.

On the contrary, it seems likely that the particles of the virus may be desiccated when deposited on the object carrier of the microscope, which illustrates what was said at the beginning of this account.

Finally the experimental inaccuracy does not permit the deduction of the existence of a hydration layer round the particle, whose thickness would be given by the difference between the value of R obtained by hydrodynamic methods on the one hand and by scattering of x-rays on the other.

It therefore seems probable, as concluded by the authors mentioned above, that the hydration of the Haricot mosaic virus might exist within the body of the particle and not at its surface. In any case the volume of water contained by the particle will be equal to the difference between

the volumes determined by the hydrodynamic methods and the dry volume determined from V_{sp}. It is found in this way that the volume of water contained is greater than the volume of the protein.

It remains to note one point which is thought to be of some importance. The quantitative data found for the particles of the virus may suggest that the latter consists of coils rather than rigid spheres.

It is obviously of great importance to solve this question. This can be done by application of the test discussed in the next section (2A).

2. EXAMPLES OF THE APPLICATION TO THE DETERMINATION OF THE SHAPE AND DIMENSIONS OF CERTAIN PARTICLES

The problem will now be limited to a real example, namely, that of a suspension of particles which are invisible in the electron microscope, and whose shape and dimensions are to be determined. It would not be a good plan, as far as this account is concerned, to give a list of the operations which can be performed and whose correct application would give a straight-forward answer to a particular problem.

Such a dogmatic method would risk giving the reader a wrong impression. Perhaps it will be applicable later when more precise knowledge has been obtained. At present it is preferable to give some examples of applications of which a critical examination will prove fruitful.

Further it will be assumed that the particles are rigid. It is indispensible, as has already been remarked several times, to verify the basis of this hypothesis and we must explain before going much further, how it is possible to decide whether a particle is effectively a rigid sphere or whether it is a molecular chain, contracted into a ball.

A. CRITERIA OF THE RIGIDITY OF A PARTICLE

It has already been stated, in the first chapter, that even when an electron photograph shows a spherical particle, it is not impossible that it may in fact be a chain macromolecule, wound round on itself whilst being precipitated on the surface of the object carrier.

Therefore it is necessary, if the ideas which are to be maintained *a priori* on the structure of the particle afford no further information, to try to dispel the uncertainty by other means.

The simplest of those which may be envisaged is the following: the solution may be made to flow with a variable velocity gradient *e.g.* between two coaxial cylinders of a Couette apparatus.

Hydrodynamics shows that the viscous frictional forces exerted on the particles produce a mutual tension along an axis of the latter (at 45° to the direction of flow for spherical particles) and a compression is the perpendicular direction (Figure 10).

If the particle is not rigid it deforms under the influence of these forces, the deformation increasing with the magnitude of the velocity gradient and the viscosity of the solvent. Now this deformation can be demonstrated experimentally, the most sensitive means being birefringence of flow.

It will be understood that, in this case, if the velocity gradient is increased gradually, the birefringence of flow will increase more quickly

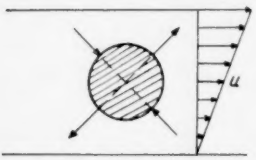


Figure 10. Tensions and pressures acting on a spherical particle in flow at constant gradient.

than if the particle were a rigid ellipsoid, since the flow progressively increases the geometrical anisotropy of the particle. The characteristics of the birefringence of flow should therefore show if the particle is not rigid. However, the effects of polydispersity can seriously affect the validity of this test and it is preferable to employ that proposed by CERF[18] of which the principle is as follows. The viscosity of the liquid containing the particles may be increased by adding a more viscous liquid which does not give appreciable birefringence of flow or by lowering the temperature.

For different values of the viscosity of the solution, the initial slope (tg α) of the curve which gives ϕ is determined as a function of the gradient G (see p. 252). If the particle is rigid, as in equation (2·12), tg α is proportional to $1/D$ and therefore according to (2·6) and (2·9) proportional to η_0. Therefore in putting the measured values for tg α as functions of viscosities η_0 of the solvent a straight line graph is obtained passing through the origin. On the contrary, calculation shows that when the particle is deformable this is no longer so.

CERF has given a striking example of this type in his experimental work.

In the case of the tobacco mosaic virus, the viscosity was increased by the addition of glycerine, and in a solution of polystyrene (chain molecule), the temperature was varied. (Figure 11).

It is clear that this test ought to be applied more often, especially to viruses.

This preliminary operation is also of value in cases where the usual birefringence test does not apply. In such cases the lack of response can be due to the fact that the dissolved particles, whilst all being elongated, are so small that their constant of rotational diffusion may be large enough to cause complete orientation in the velocity gradients normally employed

e.g. this is the case with serum albumin : only a solution to which glycerine has been added will produce a measurable birefringence[29].

If therefore the birefringence fails to appear on increasing the viscosity of the solvent, it is quite likely that the particle is not only rigid, but it is likely to be spherical, which greatly facilitates subsequent measurements.

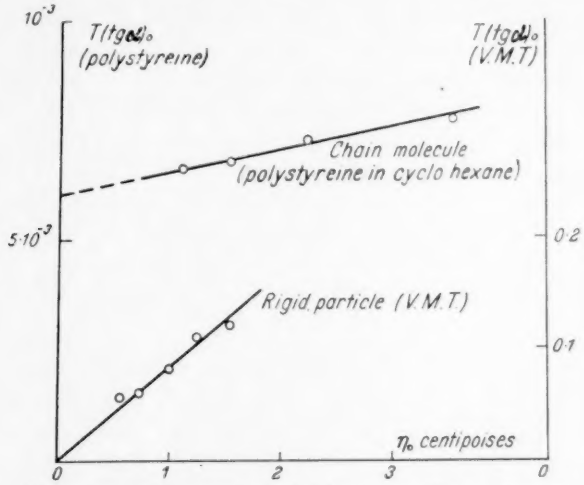


Figure 11. Difference of behaviour, according to CERF[28] between a rigid particle (tobacco mosaic virus) and a molecular chain (polystyrene in cyclohexane) when viscosity of solvent is varied.
(Ordinate : product of tan d and the absolute temperature).

Finally, if a solution does not exhibit birefringence of flow, that does not necessarily signify that it contains rigid spherical particles. It can happen that the indices of refraction of the particle and the liquid may be such that the difference between the factors g_1 and g_2 in expression (2·13) for the birefringence may be zero.

When necessary this difficulty can be overcome by modifying the nature of the solvent.

B. STUDY OF GLOBULAR PROTEINS

A concrete example will now be considered *viz.* the globular proteins, such as the albumins or the globulins, for which the electron microscope is useless. It seems likely, according to the measurements of EDSALL and FOSTER[29] on glycerine containing solutions of serum globulin, and those of SADRON, MOSIMANN and BONOT[29] on serum albumin, that the observed birefringence of flow indicates that these proteins behave as rigid particles. It is reasonable to assume that is the same for other globular proteins, but this point requires confirmation.

The second preliminary question, that of the polydispersity of the medium, is not new. It has been known since the early work of SVEDBERG that the proteins of one type seem to have a definite mass and coefficient of diffusion, as shown by their sedimentation behaviour. It is not necessary to return to these classical studies here and it will be realized at once that this is the simple case where it is known that the solution contains rigid particles which are all identical. It now remains to determine their shapes and dimensions by the general methods which have been discussed.

A considerable amount of experimental data has been obtained from a great number of different techniques for numerous proteins. It is not within the scope of this account to give a detailed table and criticism of all such results*. The discussion will be limited to a single example, namely that of horse serum albumin, since the same principles are applicable to other proteins, apart from differences relating to detail.

In the following table the principal experimental data for aqueous solutions have been collected. The values have all been reduced to 20°C*.

Table 1—Horse Serum Albumin
(Value for pure water at 20°C)

S	A	M_s	M_e	M_{osm}	M_{es}	$[\eta_i]$	V_{sp}	R_o	D
$4.5.10^{-13}$(*a*)	$6.17.10^{-7}$(*c*)	70.100(*i*)	68.000(*j*)	65.000(*k*)	76.500 ; (5780 Å)	4.88(*n*)	0.748(*n*)	23 Å(*o*)	9.000 sec−1 (*q*)
4.4—(*b*)	6.45—(*d*)	67.100(*d*)	67.000(*b*)	72.000(*l*)	(*m*) 72.000	4.3 ; 4.28(*e*)		27 Å(*p*)	
	6.83—(*e*)				(5461 Å)	3.80			
	7.24—								$\frac{D\,\eta_0}{T} = 61$
	6.9—(*f*)								
	6.83—								
	6.5—(*g*)								
	6.1—(*h*)								

(*a*) P. VON MUTZENBECHER; *Bioch. Z.* 266 (1933) 226, 250, 259.
(*b*) THE SVEDBERG and K. O. PEDERSEN; *Die Ultrazentrifuge* p. 337. Th. Steinkopf, Dresden und Leipzig 1940
(*c*) A. POLSON; *Koll. Z.* 87 (1939) (2) 163.
(*d*) O. LAMM and A. POLSON; *Bioch. J.* 30 (1936) 528.
(*e*) H. NEURATH, G. R. COOPER and T. O. ERICKSON; *J. biol. Chem.* 138 (1941) 411
(*f*) KECKWICK, ; *Bioch. J.* 32 (1938) 552
(*g*) M. CHAMPAGNE; à paraitre *J. chimic. Phys.* (1952)
(*h*) R. A. ALBERTY; *J. Amer. Chem. Soc.* 70 (1948) 1675
(*i*) A. POLSON; *Koll. Z.* 87 (1939) (2) 178
(*j*) THE SVEDBERG and K.O. PEDERSEN; *Die Ultrazentrifuge* p. 368 — *cf* (b)
(*k*) A. G. OGSTON; *Bioch. J.* 31 (1937) (II) 1952
(*l*) G. S. ADAIR, M. E. ROBINSON; *Bioch. J.* 24 (1930) (II) 1864
(*m*) PUTZEYS, P. and BROSTEAUX, J. ; (1941)
(*n*) A. POLSON; *Koll. Z.* 88 (1939) 58
(*o*) G. FOURNET; *Bull. Soc. Franc. Min. et Cristall* T. LXXIV (1951) p. 37
(*p*) H. N. RITLANG, P. KAESBERG and W. W. BEEMAN; *J. Chem. Phys.* 18 (1950) 1237
(*q*) EDSALL, J. T. and J. F. FOSTER; *J. Amer. Chem. Soc.* 70 (1948) 1860

* In this table the symbols already used have their usual significance. The others are as follows :
R_g = radius of gravation.
M_s = mass of the particle obtained from s and A
M_e = mass of the particle obtained from sedimentation equilibrium.
M_{osm} = mass of the particle obtained from osmotic pressure.
$M_{l.s.}$ = molar mass of the particle obtained from the Rayleigh constant.

A large number of experimental results are available and this constitutes a point of which the consequences will be seen later. The lack of accuracy of measurements, or the consistency of preparation, is such that variation in the masses amounts to $\pm$ 10% for Δ and R_0, and to more than $\pm$ 10% for the intrinsic viscosity.

It is readily apparent in these conditions that the experimental data must be interpreted very carefully, since when suitably handled they can verify whatever theory may be desired.

This lack of accuracy in the calculated dimensions will appear in the course of the brief analysis which will be made according to the principles already stated (see IIc., p. 255).

It will be supposed here that the mass is known and it will be taken equal to the mean of the molar masses measured, which is 69,000, giving the mean molecular mass as

$$M = 11.5.10^{-20}$$

The remaining unknowns are the lengths a and b of the semi-axes of the ellipsoid of revolution (or of the cylinder of revolution) equivalent to the hydrodynamic particle of serum albumin, or its axial ratio p, and its volume V. Experiment gives numerical values from expressions for $\bar{f}$ (from S or Δ) $[\eta]$ and D, containing these two parameters.

In principle, if the measurements were sufficiently accurate, comparison between these diverse numbers would indicate whether the particle of serum albumin was lacunar, and permit calculation of the order of magnitude of the hydration film which may surround it. It will be seen that, unfortunately, the experimental results are still insufficient for it to be possible to obtain more than vague indications from the measurements.

1. *Dimensions of the hydrodynamic particle*

From values of $\bar{f}$,$[\eta]$ and D, those of p and V can be obtained from three possible combinations :
$\bar{f}$ and $[\eta]$, D and $[\eta]$, and $\bar{f}$ and D, the latter combination having the advantage of not involving the mass.

(*a*) *Combination of* $\bar{f}$ *and* $[\eta]$—From expressions for $\bar{f}$ (equation 2·8), and from $[\eta]$ (equation 2·17) after eliminating V;

$$\Lambda\ t^3 = \left(\frac{6\pi\ \eta_0}{1{\cdot}612\,\bar{f}}\right)^3 M\,[\eta] \qquad \ldots\ (6{\cdot}1)$$

All the magnitudes appearing in the second part of this equation are known so that the value of $\Lambda\ t^3$ may be calculated, which gives that of p. Having p, $\Lambda\ (p)$ is obtained from tables, and V is calculated from the equation $V = \dfrac{M\,[\eta]}{\Lambda\,(p)}$

In the case of serum albumin, sedimentation gives :

$$\bar{f} = \frac{1}{S}(1 - V_{sp} \cdot \rho) \cdot M = 0{\cdot}656.10^{-7} \qquad \ldots . (\alpha)$$

The constant of translational diffusion gives :

$$\bar{f} = kT/\Delta = 0{\cdot}606.10^{-7} \qquad \ldots . (\beta)$$

where the mean of the values for Δ appearing in the table is taken as $\Delta = 6{\cdot}6.10^{-7}$.

Hence equation (6·1) gives :

1. with $\bar{f} = 0{\cdot}656.10^{-7}$, $At^3 = 3{\cdot}16$ whence $p = 4{\cdot}2$,

and $\triangle = 7{\cdot}45$ and, from the value $[\eta] = 4{\cdot}88$:

we obtain $V = 750.10^{-18}$cc.

2. with $\bar{f} = 0{\cdot}606.10^{-7}$, $At^3 = 3{\cdot}96$ whence $p = 11{\cdot}8$,

$\triangle = 17{\cdot}7$: $V = 640.10^{-18}$ cm^3.

When it is remembered that the volume of the 'dry' particle calculated from V_{sp} is :

$$V = V_{sp}\, M = 860.10^{-18} \text{ cc}$$

it will appear that the volume of the hydrodynamic particle is smaller than that of the dry particle.

On the contrary, if instead of taking the value of 4·88 for $[\eta]$, the value 4·3 is taken corresponding to the second set of experiments figuring in the table, p and V are found according to the two values chosen for $\bar{f}$:

$\bar{f} = 0{\cdot}656.10^{-7}$ $\quad p = 4{\cdot}55$ $\quad V = 940.10^{-18}$ cc
$\bar{f} = 0{\cdot}606.10^{-7}$ $\quad p = 7{\cdot}90$ $\quad V = 500.10^{-18}$ cc

of which only the former is greater than the volume of the dry particle.

This simple example shows how large is the uncertainty for the calculated values of the axial ratio and the volume of the ellipsoid equivalent to the molecule of serum albumin.

This uncertainty depends in the first place on the inaccuracy of $\bar{f}$. Whilst errors in the values of S and Δ are considerable (α and β), it will be noticed that f appears to the third power in (6·1).

The situation is obviously not improved by putting the value of V obtained from V_{sp} in the expression for f as is often done. Another error is added by this method, in which the volume of the hydrodynamic particle is greater than that of the dry particle.

(*b*) *Combination of D and* $[\eta]$—Eliminating V between the expressions for D (2·6 and 2·9) and $[\eta]$ gives :

$$r(p)A = \frac{D[\eta]}{kT} 6\eta_0 M \qquad \ldots . (6\cdot2)$$

This equation, with the expression for $[\eta]$ can be used in the same way as equation (6·1) in the preceding paragraph. Taking EDSALL and FOSTER'S value of 61 for $D\eta_0/T$ the following values are obtained :

with $[\eta] = 4\cdot88$, $rA = 1\cdot986$ whence $p = 3\cdot3$ and $V = 14\cdot2.10^{-20}$ cc
with $[\eta] = 4\cdot3$, $rA = 1\cdot754$ whence $p = 4\cdot45$ $V = 9\cdot6.10^{-20}$ cc

The volumes obtained are in this case, clearly greater than the volume of the dry particle and the values of p are also compatible with those given by ONCLEY[30] which were obtained by a method which has not been described and which is to be found elsewhere.*

It is necessary to note that the method relying on the determination of D and $[\eta]$ is in principle preferable to the method based on $\bar{f}$ and $[\eta]$, since it uses the measured value of D at the first power.

The accuracy required for the measurement of D is less than that necessary for $\bar{f}$ *i.e.* than that for S or Δ.

Unfortunately, in the present case, it is very difficult to make a correct measurement of D since the birefringence of serum albumin solutions is very feeble, even in the presence of glycerine. The values of $D\eta_0/T$ given by EDSALL and FOSTER are not known with certainty to within less than 25 per cent.

(*c*) *Combination D and* $\bar{f}$—Elimination of V from expressions for D and f gives :

$$rt^{-3} = \frac{D\bar{f}^3}{{\eta_0}^2} \cdot \frac{1}{kT} \cdot \frac{1}{27\pi^2} \qquad \ldots . (6\cdot3)$$

This expression associated with the expression for D or $\bar{f}$ gives the values of p and V.

This method possesses the same difficulties as that involving $\bar{f}$ and $[\eta]$ and, in the present case, there is the additional complication of the delicate measurement of D.

On the contrary, it does not involve the mass of the particle.

(*d*) *Recapitulation*—It will be useful to give a list of the pairs of values of p and V which have been obtained from the preceding analysis and which appear in the first two lines of Table I.

It will be recalled that these numbers have been calculated on the assumption that the particle is a long ellipsoid. It would be necessary, to be rigorous, to give with these numbers those found on the *a priori* assumption of an elongated cylinder of revolution, an ellipsoid, or a flat

* General literature.

cylinder of revolution. It is not necessary to give the results of this calculation here. In fact these only become of interest when the dispersion of experimental data is small enough for comparison of numbers found for different shapes, so that a systematic choice may be made between them.

2. *Dimensions of the particle by non-hydrodynamic methods*

In the course of the preceding section the volume of the dry particle: $V = 860.10^{-18}$ cm^3 has already been compared with that of the hydrodynamic particle. Now follow the dimensions determined from the radius of gyration.

If it is supposed that the particle is an ellipsoid of revolution, whether elongated or flattened,

$$R_0^2 = (b^3 + 2a^3)/5a \qquad \ldots . (6{\cdot}4)$$

and again

$$R_0^2 = \left(\frac{3}{4\pi}V\right)^{2/3} \frac{1 + 2p^3}{5p^{5/3}} \qquad \ldots . (6{\cdot}5)$$

whence approximately:

$$p = 4\frac{R_0^{3/2}}{V^{\frac{1}{2}}} \qquad \ldots . (6{\cdot}6)$$

If the general hypothesis is maintained that the particle which scatters x-rays is neither the dry particle, nor the hydrodynamic particle, the investigations cannot be pushed any further. What will be done here therefore will be to substitute for V the different values found above for the hydrodynamic particle and to calculate the value of p which gives the radius of gyration. The result of this operation is contained in the last two lines of Table II.

Table II

p	6·2	11·8	4·55	7·9	3·3	4·55
$V\ 10^{18}$ (in cc)	750	640	940	500	1420	960
$R_0 = 27$ Å $p =$	20·4	22·1	18·3	25	14·8	18
$R_0 = 23$ Å $p =$	14·7	16	13·2	18·0	10·7	13·0

It is seen that the values of p found in this way are systematically two or three times greater than those given by hydrodynamic measurements.

3. *Conclusions*

From the numbers appearing in Table II it may be concluded that the information obtained from the methods which have been applied are still very vague.

In particular the results are too discordant to provide even any approximate indications of the hydration of the molecule of serum albumin. It is not possible at this stage to say whether it is long or flat.

All that can be affirmed is that it is a non-spherical particle of which the geometrical anisotropy is not very great and whose volume is of the order of 900.10^{-18} cm^3.

Nevertheless, the poorness of these results is not due to the inefficiency of the methods whose use has been described. It is necessary in fact to note that the data collected together in Table I belong to markedly differing preparations. To obtain a true impression of the value of the measurements it would be necessary, obviously, to compare the results for the same preparation. It is certain that discussion of the numbers obtained would lead to more reliable and consistent conclusions.

This explains also, how many authorities obtain more detailed and more affirmative results from their experiments than those which have been given above. It is thought necessary, however, for this review to remain in a prudent position of doubt.

C. STUDY OF SOLUTIONS OF DEOXYRIBONUCLEIC ACID (IN THE REGION OF NEUTRALITY)

A rapid examination of the properties of solutions of desoxyribonucleic acid, or rather, of its sodium salt, such as the viscosity or the birefringence of flow, shows that they depend on two principal factors; the ionic strength of the solution, and the *p*H[31].

The study of the influence of *p*H, which is still far from being elucidated, will be omitted, and only solutions in the neutral region will be discussed, in which small variations of *p*H seem to have no effect.

On the contrary the ionic strength must be mentioned, at the risk of slightly complicating this account, because of the importance of the conclusions to be drawn from it.

1. *Effect of the concentration of sodium chloride in neutral solution. Critical concentrations*

Experiment shows that an aqueous solution of thymonucleic acid has a large specific viscosity and also a marked birefringence of flow, but these characteristics are abruptly diminished by the addition of a small quantity of sodium chloride to the solution.

Such a phenomenon can be attributed, *a priori*, to two principal causes :

1. The particle of nucleic acid is modified by the addition of the salt : *e.g.* its geometric anisotropy may be diminished in such a manner that the birefringence of flow as well as the value of $\Delta\,(p)$ (and of $[\eta]$) may be lowered considerably.

2. The particle remains unchanged but the electrostatic interaction between these particles can be considerably decreased by the introduction of the electrolyte. In this case the observed effect will decrease when the concentration of the acid is decreased and, in the limit, for $c \to o$, the effect will be zero.

Hence it is easy, in principle, to distinguish between the two hypotheses *e.g.* by the comparison of intrinsic viscosities $[\eta]$ of the nucleic acid solution in the presence and absence of NaCl. The intrinsic viscosity, which depends solely on the shape of the particle and its dimensions and

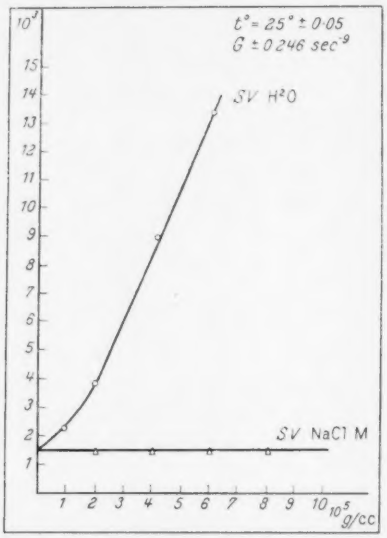


Figure 12. Specific viscosity in sodium thymonucleate solutions, at zero gradient, as function of concentration
○ in pure water
△ in 10% aqueous sodium chloride.
The two curves are tangential at $C = o$, indicating that $[\eta]$ is the same in the two cases (after POUYET[32]).

not on its interactions, should remain unchanged by the addition of sodium chloride. POUYET[32] has found this whilst studying the thymonucleate prepared by SIGNER and SCHWANDER[34] with the Couette viscometer. The results of the measurements are shown in Figure 12 which shows clearly within the experimental accuracy that the limit of η_{sp}/c when $c \to o$ is the same for two solutions of which the one is in pure water and the other is in water containing 10 gm of NaCl per 100 cc.

It is seen, on the contrary, that the viscosity of the solutions in water increases considerably as a function of the concentration of the nucleic acid, which indicates the existence of very strong electric interactions. In fact experiment shows that for two SIGNER preparations, SV and SVIII* the measurements of the viscosity in pure water become non-reproducible

* SV prepared with sodium fluoride as inhibitor; SVIII with sodium citrate (the latter probably contains larger particles).

when the concentration is greater than two to three milligrams of nucleic acid per 100 cc for SV and one to two milligrams for SVIII. These concentrations seem to be critical concentrations above which solution without added salt is no longer a real liquid.

Only below these critical concentrations does one obtain true solutions to which the general theories which have been discussed are applicable. On the contrary the chloride solutions are not at all troublesome and can be considered as true solutions at concentrations ten times greater.

It has been necessary to show these preliminary results before passing to the study of the morphology of the particles of nucleic acid in solution : a sodium chloride solution will be considered whenever possible in what follows, since it has the advantage of not only avoiding the existence of difficult types of interactions, but further of avoiding the rapid degradation which is sometimes observed in purely aqueous solutions.

2. *Is the nucleic acid particle rigid?*

According to its chemical structure the particle of sodium thymonucleate is a long chain consisting of complex bases united through a chain of alternate phosphate and sugar (deoxy-ribose) groups[35] (Figure 13).

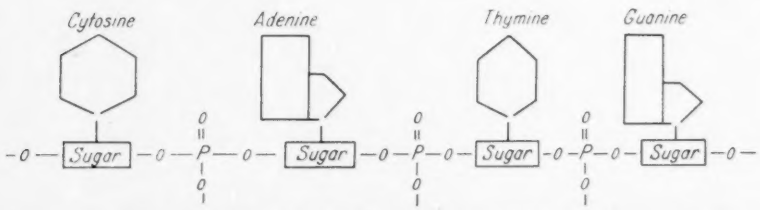


Figure 13. Scheme of part of the nucleic acid chain. In principle rotations can occur round the oxygen atoms which are linked to the sugar groups, but they may be prevented by the steric hindrance of the side groups, or by interactions between them.

We propose here not to discuss the nature of the proportions and of the relative order of the purine bases but simply to note that if such a structure exists, it is possible for rotations to occur round the bonds which unite the atoms constituting the skeleton of the chain. Consequently, the nucleic acid molecule cannot possess a unique configuration, or even several well defined configurations, but an infinity of configurations which change with thermal agitation.

For this reason SCHWANDER and CERF[36] have tried to apply the test of birefringence for deformable particles to this case by changing the viscosity of the solution (containing sodium chloride) by the introduction of glycerine.

Figure 14 shows the values of tg α obtained for different viscosities. The first four points lie on a straight line passing through the origin, as is typical of a rigid particle.

However, the two points corresponding to the highest viscosities deviate considerably from this line.

As the value corresponding to the birefringence is abnormally low for these viscosities it seems reasonable to suppose that the heating produced

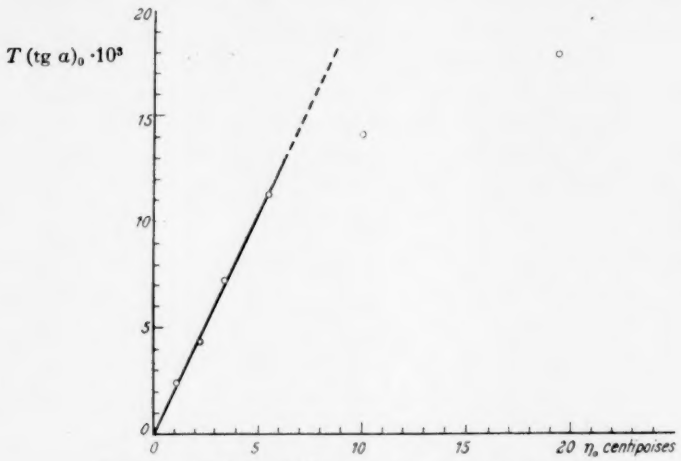


Figure 14. Values of T (tg α)$_0$ for a solution of sodium thymonucleate with sodium chloride, when viscosity is varied by addition of glycerine. At the value $\eta_0 = 7$ centipoises, the nucleic acid behaves as a rigid rod (after CERF and SCHWANDER[36]).

by the friction would have been sufficiently high to cause a noticeable reduction in viscosity, which would explain the deviation of these two points. Nevertheless their existence means that it cannot be concluded with complete certainty that the molecule of nucleic acid is quite rigid, but this idea remains the more likely however, and it is confirmed by other methods.

3. *Polydispersity of solutions of nucleic acid*

In the case under investigation nothing is known about the monodispersity of the samples.

The study of the sedimentation of the solution of such great dilutions involves great difficulty. In fact, all experiments carried out in this field[37] have been made at such high concentrations that any interpretation is impossible.

On the contrary, the methods of Brownian diffusion such as have been described early (p. 269) are applicable; in particular BENOIT[33] has used

the Kerr effect to study the SV preparation of nucleic acid dissolved in pure water. He found in this way that the solution behaved as though it contained about 30 per cent of particles of which the rotational diffusion constant was equal to 400 sec.$^{-1}$ and 70 per cent whose diffusion constant was equal to 1,150 sec.$^{-1}$ (see Figure 8). It is quite obvious that BENOIT's experiments have never pretended to imply that the solution contained *only* two types of particles; they show that within the resolving power of the method the solution behaved as a mixture of two types, though it might contain a large number.

4. *Study of the morphology of nucleic acid particles*

Knowing now that the solution contains rigid particles, whose shape does not depend on the sodium chloride content, and that the solution is polydisperse, the results of measurements made on these solutions will be discussed. In the first place certain of the measurements will be briefly described.

(*a*) *Birefringence of flow*

The results obtained by SCHWANDER and CERF[38] are shown in the diagram of Figure 15. From the appearance of these curves and from the magnitude of the birefringence, it can be deduced that it is extremely probable

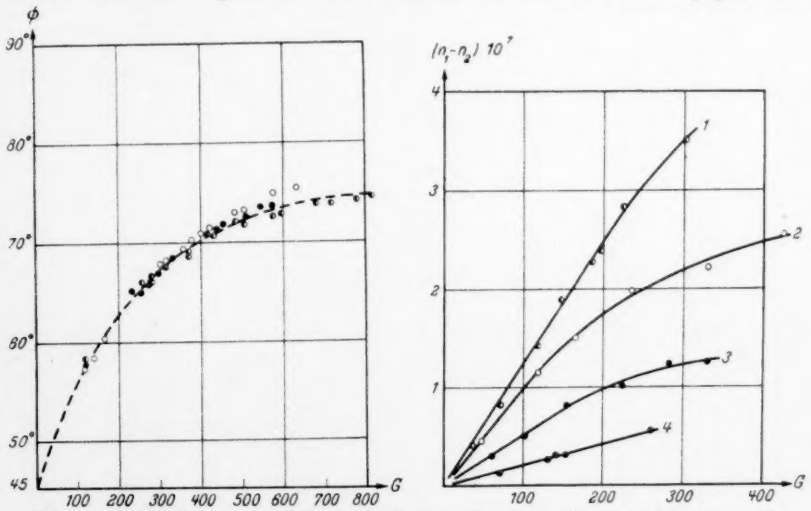


Figure 15. Birefringence of flow of solution of sodium thymonucleate in 10% aqueous sodium chloride

- 19·1 mg. nucleate/100 cm^3
- 14·7
- 10·3
- 0·9

(after SCHWANDER and CERF).

that the particles are very long, and there is no suggestion that the particle is deformable, which confirms the preceding conclusions.

It is found for the chloride solutions that the curve ϕ (G) is practically independent of the concentration of the nucleic acid when the latter is less than about 0·02 per cent.

The corresponding constant of rotational diffusion is $D = 37$ sec.$^{-1}$.

As the solution is polydisperse this value is a mean, but it is noticeable that it is not of the order of magnitude of the constants of diffusion measured by the Kerr effect. This constitutes an anomaly whose origin should be investigated but it is not impossible that it may be explained by the different physical phenomena on which the measurements depend.

(*b*) *Intrinsic viscosity*

The viscosity of a solution of nucleic acid, even in the presence of sodium chloride, depends essentially on the speed of flow of the solution in the capillary, as different authorities have shown, particularly VALLET and SCHWANDER[39]. The existence of such a phenomenon indicates either that the particle is deformed in the velocity gradient due to the flow or whether being quite indeformable, it orientates itself in the flow because of its geometrical anisotropy.

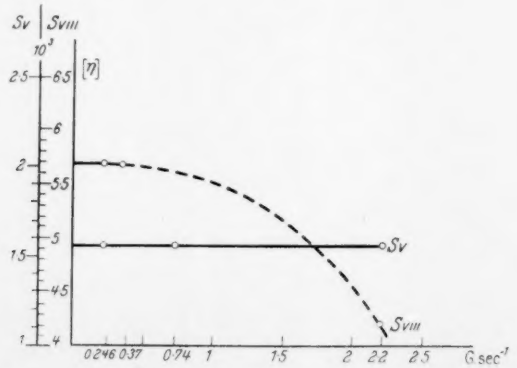


Figure 16. Intrinsic viscosity of two sodium thymonucleate solutions, SV and SVIII as function of gradient in Couette viscometer (after POUYET[40]).

The first hypothesis is rejected for the reasons already given (birefringence of flow). There remains however the second one, which is perfectly consistent with a model of a long particle. In fact, in this case the orientation of the rod occurs readily with the result that 'structural' viscosity is observed, as was mentioned previously (p. 254).

In order to obtain a value for the intrinsic viscosity corresponding to formula (2·17), from this it is necessary to work at zero gradient.

For this reason POUYET[40] carried out his measurements of viscosity in the Couette viscometer, which has the advantage over the capillary viscosity of realising a uniform velocity gradient throughout the liquid. The sensitivity of the instrument is sufficient for accurate measurements to be made for solutions of such low concentrations as 1 mg per 100 cc, with gradients of the order of 0·246 sec^{-1} *i.e.* a thousand times smaller than the gradients generally obtained by the capillary methods. At these very small gradients the orientation effect is negligible for the SV preparation, but it still exists for the SVIII preparation (Figure 16).

Extrapolation to zero gradient at 25°C gives :

for SV: $[\eta]$ = 1500 c.g.s.; for SVIII : $[\eta]$ = 5700 c.g.s.

It has already been seen that these values of $[\eta]$ are independent of the concentration of sodium chloride.

Obviously they constitute mean values.

(*c*) *Light scattering*

Light scattering by nucleic acid solutions has been studied intensively by different authorities[41]. In this case marked dissymmetry clearly exists.

The values obtained for $Z = I_{45°}/I_{135°}$ and for M are as follows (all in NaCl, 0·2 M; and at *p*H7)

Z = 3·1 for a solution of preparation SV.
M = 3.200.000 by dissymmetry (OSTER).
Z = 3·8 for a 'Bunce' sample.
M = 2.310.000 by dissymmetry (B. BUNCE).
M = 4.000.000 by extrapolation.
Z = 4·1 for sample of SVIII (B. BUNCE).
M = $6{\cdot}7.10^6$ (by extrapolation).

(*d*) *Discussion of results*

Shape of the particle. The experiments on birefringence of flow and on viscosity show that the nucleic particle behaves as if they were rigid and fairly anisodiametric.

But it is known that, according to DEBYE[42], the dissymmetry observed in the case of long rods cannot exceed 2·4 (Section II, 3B). The measurements of scattered light, which give distinctly greater values of the dissymmetry, are in contradiction with the above assumption. However when the anisotropy is taken into consideration, it is seen that the dissymmetry can attain the value 3·4[11]. In this case the contradiction disappears for the sample of SV, whilst it is less clear for the 'Bunce' sample, and it remains marked for the SVIII sample.

Perhaps it will be possible to reconcile the necessarily lengthy shape of the particle with the data from dissymmetry of the light if it is assumed

that the solution contains a mixture of straight and kinked rods. Or again the rod may bear branches of important dimensions.

Dimensions of the particle. It is clear that, not being certain of the shape of the particle, its dimensions cannot be calculated.

It will therefore be assumed, in accordance with the general hypothesis, that the particles are all straight rods. Also it will be assumed that the section of the rod is of the order of 100 Å as follows from the researches on x-ray diffraction[43].

In these conditions, knowing b, the value of a can be obtained from that of the constant of rotational diffusion. It has been found from the measurements of the Kerr effect by BENOIT that the two constituents of the SV sample have half lengths :

$$\left.\begin{array}{ll} a = 1900 \text{ Å} & \\ & \text{and axial ratios} \\ a = 1350 \text{ Å} & \end{array}\right\} \begin{array}{l} p = 380 \\ \\ p = 270 \end{array}$$

On the contrary, measurement of D by birefringence of flow leads to a mean value :

$a = 400$ Å with an axial ratio $p = 800$.

If now it is supposed—an extremely doubtful hypothesis since the least hydration of the rod changes its hydrodynamic volume considerably—that the hydrodynamic is sensibly equal to the 'dry' volume, the ratio V/M in the expression for the viscosity can be replaced by the value of the partial specific volume V_{sp}. Whilst it is true that this quantity is not well established, it will be assumed in conformity with KAHLER[37] that it is equal to 0·56.

The intrinsic viscosity of the mixture of the two constituents detected by the Kerr effect is :

$$[\eta] = V_{sp} [\nu_1' \, A(p_1) + \nu_2' \, A(p_2)]$$

It has been seen that :

$$\nu_1' = 1/3; \quad \nu_2' = 2/3; \quad p_1 = 380; \quad p_2 = 290$$

In conjunction with corresponding values of $A(p)$ the value of $[\eta]$ may be calculated as :

$$[\eta] = 1700$$

which is of the same order, 1500, as that measured by POUYET.

It is possible that this agreement is only a coincidence and it should not be regarded very highly, at least not as an important proof. Also it seems difficult to admit the pure and simple piling up of the nucleotides because of the steric hindrances, so that other shapes seem more probable[44].

But if the hypothesis that the particles of the SV solution are rigid rods having the dimensions already given, is maintained, another contradiction is reached. In fact if reference is made to the studies made by

ASTBURY[43] on the diffraction of X-rays by the fibres of nucleic acid it is found that the rod is a pile of nucleotides whose separation is 3·3 Å; a rod of 2000 Å in length will then contain about 600 nucleotides. The mass of a nucleotide being 330, the mass of the particle will definitely be less than 350,000. Now for the SV sample the mass measured by light scattering is almost ten times larger. Perhaps it is best not to attach too much importance to this difference since the mass which has been calculated from the length of the rod represents a numerical mean, different from the mean obtained from the light scattering. In any case the least that can be said is that it is prudent to remain in doubt.

This is, in addition, the conclusion which is required from the whole of experiments which have been outlined. All that can be advanced with any certainty is that the sodium thymonucleate in solution forms rigid and very anisodiametric particles, which are responsible for the birefringence of flow at small velocity gradients and that it is possible that it forms also particles of another form, much less elongated than the first and perhaps even coils. Such particles would be responsible for the great value of the dissymmetry of diffused light. According to the method of measurement, one observes chiefly particles of either the one or the other kind.

5. *Conclusion*

The author has desired, in choosing proteins and the nucleic acids, to give examples of the applications of general methods which would yield new results for the structures of the particles.

Although the results obtained are vague, it will be possible—the author hopes—to assert from these examples that if the methods described are less easy to use than some think they can nevertheless be very effective.

It is necessary, in order that satisfactory results may be obtained in this field, that much more work should be performed to increase the accuracy and the sensitivity of the physical measurements.

It is equally necessary to be much more sure of the legitimacy of the formulae which the theories have produced than is the case at present, particularly in the field of 'small macromolecules'.

Also it is necessary that the results obtained may be compared with those, more accurate and numerous, which specialists in structure analysis obtain from spectral methods. Finally no progress can be made unless biochemists also improve their methods of preparation so as to isolate definite and reproducible substances. But that, it may be said, will come from the common efforts of chemists and physical chemists, and will be the result of new knowledge.

APPENDIX

I. FRICTIONAL COEFFICIENTS FOR AN ELLIPSOID (from F. PERRIN)

Lengths of the semi-axes : a_1, a_2, a_3.

(*a*) *Translation.* f_1, f_2, f_3 frictional coefficients for translations respectively parallel to a_1, a_2, a_3.

Let :

$$P = \int_0^\infty ds/(a_1^2 + s)\ [\ (a_1^2 + s)\,(a_2^2 + s)\,(a_3^2 + s)\ ]^{1/2}$$

$$Q = \int_0^\infty ds/(a_2^2 + s)\ [\ (a_1^2 + s)\,(a_2^2 + s)\,(a_3^2 + s)\ ]^{1/2}$$

$$R = \int_0^\infty ds/(a_3^2 + s)\ [\ (a_1^2 + s)\,(a_2^2 + s)\,(a_3^2 + s)\ ]^{1/2}$$

We have :

$$P + Q + R = 2/a_1, a_2, a_3$$

$$a_1^2 P + a_2^2 Q + a_3^2 R = S$$

Then :

$$f_1 = 16\pi\eta_0/(S + a_1^2 P);\ f_2 = 16\pi\eta_0/(S + a_2^2 Q);\ f_3 = 16\pi\eta_0/(S + a_3^2 R)$$

If the ellipsoid is of revolution :

$$a_1 = a,\ a_2 = a_3 = b,\ Q = R$$

$$f_1 = 16\pi\eta_0\,(a^2 - b^2)/[(2a^2 - b^2)\,S - 2a]$$

$$f_2 = f_3 = 32\pi\eta_0\,(a^2 - b^2)/[(2a^2 - 3b^2)\,S + 2a]$$

with :

$$S = \int_0^\infty ds/(b^2 + s)\,(a^2 + s)^{1/2}$$

If $a > b$ (elongated ellipsoid)

$$S = 2\,(a^2 - b^2)^{-1/2}\ \ln\ \{[a + (a^2 - b^2)^{1/2}\ ]/b\} \qquad \ldots\ (1)$$

If $a < b$ (oblate ellipsoid)

$$S = 2\,(a^2 - b^2)^{-1/2}\ \text{Arc tg}\ [(b^2 - a^2)^{1/2}\ /a] \qquad \ldots\ (2)$$

If $a = b$ (sphere)

$$f_1 = f_2 = f_3 = 6\pi a\eta_0 = \frac{1}{1{,}612}\,6\pi\eta_0 V^{1/3}$$

The mean frictional coefficient $\bar{f}$ for a random distribution of the orientation of the ellipsoid during its translation is given by :

$$\frac{1}{\bar{f}} = \frac{1}{3}\left(\frac{1}{f_1} + \frac{1}{f_2} + \frac{1}{f_3}\right)$$

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If the ellipsoid is of revolution (volume V, elongation p) one may write

$$f = \frac{1}{1{,}612}\, 6\,\pi\,\eta_0\, V^{1/3}\, 1/t\,(p) \qquad \ldots\ (3)$$

where $t\,(p) = 1$ for $p = 1$.

The general expressions for $t\,(p)$ are:

$$p > 1 \begin{cases} t\,(p) = \dfrac{p^{1/3}}{\sqrt{p^2 - 1}} \ln\left[p + \sqrt{p^2 - 1}\right] \\ \text{if } p > 50\ ,\ t\,(p) = p^{-2/3} \ln 2\,p \end{cases} \qquad \ldots\ (4)$$

$$p < 1 \begin{cases} t\,(p) = \dfrac{p^{1/3}}{\sqrt{1 - p^2}} \operatorname{Arc\,tg} \dfrac{\sqrt{1 - p^2}}{p} \\ \text{if } p < 1/50\ ,\ t\,(p) = p^{1/3} \operatorname{Arc\,tg} 1/p \end{cases} \qquad \ldots\ (5)$$

It may be useful to note that equation (3) can be written simply:

$$f = 6\,\pi\,\eta_0\, b\, \frac{p^{1/3}}{t\,(p)} \qquad \ldots\ (6)$$

This relation holds for a cylinder of revolution fairly elongated or fairly flattened. In that case, since $V = \pi\, p\, b^3$

$$f = 6\,\pi^{2/3}\,\eta_0\, \frac{V^{1/3}}{t\,(p)} \qquad \ldots\ (7)$$

(*b*) *Rotations.* C_1, C_2, C_3 friction coefficients for rotations respectively around a_1, a_2, a_3.

In the case of an ellipsoid of revolution ($a_1 = a$, $a_2 = a_3 = b$)

$$C_1 = \frac{16\,\pi}{3}\,\eta_0\,(a^2 - b^2)\, b^2/(2a - b^2 S)$$

$$C_2 = C_3 = \frac{32\,\pi}{3}\,\eta_0\,(a^4 - b^4)/[(2a^2 - b^2)\, S - 2a]$$

S being given by equations (1) or (2) according to the case (elongated or oblate ellipsoid).

If $a_1 = a_2 = a_3 = a$ (sphere)

$$C_1 = C_2 = C_3 = 8\,\pi\, a^3\,\eta_0 = 6\,\eta_0\, V \qquad \ldots\ (8)$$

If V is the volume of the ellipsoid and p its elongation, one may write :

$$C_2 = C_3 = C = 6\,\eta_0\,V\,\frac{1}{r\,(p)} \qquad \ldots\ldots (9)$$

where $r\,(p) = 1$ for $p = 1$

Then the function $r\,(p)$ is given by the following equations :

$$p > 1 \begin{cases} r\,(p) = \dfrac{3}{2}\,\dfrac{p^2}{p^4 - 1}\left[-1 + \dfrac{2\,p^2 - 1}{p\,\sqrt{p^2 - 1}}\,\ln\left(\dfrac{p + \sqrt{p^2 - 1}}{p - \sqrt{p^2 - 1}}\right)\right] \\ \text{If } p \text{ is larger than about 50 :} \qquad \ldots\ldots (10) \\ r\,(p) = \dfrac{3}{p^2}\,[\ln 2\,p - 0,5] \end{cases}$$

$$p < 1 \begin{cases} r\,(p) = \dfrac{3}{2}\,\dfrac{p^2}{1 - p^4}\left[1 + \dfrac{1 - 2\,p^2}{p\,\sqrt{1 - p^2}}\,\text{Arc tg}\,\dfrac{\sqrt{1 - p^2}}{p}\right] \\ \text{If } p \text{ is smaller than } 1/50 \qquad \ldots\ldots (11) \\ r\,(p) = \dfrac{3}{2}\,p^2\left[1 + \dfrac{1}{p}\,\text{Arc tg}\,\dfrac{1}{p}\right] \end{cases}$$

In the case of a cylinder of revolution fairly elongated or fairly flattened, we have :

$$C_2 = C_3 = C = \frac{2}{3}\,\eta_0\,V\,\frac{1}{r\,(p)} \qquad \ldots\ldots (12)$$

with the same values of $r\,(p)$ as above.

II. INTRINSIC VISCOSITY OF A SUSPENSION OF ELLIPSOIDS OF REVOLUTIONS OR OF CYLINDRIC RODS

(*a*) *Ellipsoid.*

$$[\eta] = \frac{V}{M}\,\Lambda\,(p) \qquad \ldots\ldots (13)$$

M is the molecular weight of the solved particle, V and p respectively the volume and axial ratio of the hydrodynamic particle. If $p = 1$ (sphere); $\Lambda = 2{\cdot}5$.

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If the gradient of velocity is so small that there is no noticeable effect of orientation :

$$p > 1 : \; A(p) = \frac{p^2}{15} \frac{1}{\ln 2p - 1{\cdot}50} + \frac{p^2}{15} \frac{1}{\ln 2p - 0{\cdot}50} + \frac{14}{15} \quad \ldots . (14)$$

When p is larger than about 50 :

$$A(p) = \frac{4p^2}{15} \frac{1}{\ln 2p} \quad \ldots . (15)$$

$p < 1$: and smaller than 1/50

$$A(p) = \frac{16}{15} \frac{1}{p} \text{ Arc tg } \frac{1}{p} \quad \ldots . (16)$$

(*b*) *Cylinder* (*elongated or fairly flattened*).

$$[\eta] = \frac{2}{3} \frac{V}{M} A(p) \quad \ldots . (17)$$

$$\text{if } p > 1 : \; A(p) = \frac{p^2}{15} \frac{1}{\ln 2p - 1{\cdot}80} + \frac{p^2}{5} \frac{1}{\ln 2p - 0{\cdot}80} + \frac{14}{15}$$

if $p < 1$: same equation as 16.

III. MAXWELL COEFFICIENTS.

$$L_1 = \frac{4\pi}{3}(1 - 2\alpha)$$

$$L_2 = \frac{4\pi}{3}(1 + \alpha)$$

with

$$\alpha = \frac{1}{4(p^2 - 1)} \left[2p^2 + 4 - \frac{3p}{\sqrt{p^2 - 1}} \ln \frac{p + \sqrt{p^2 - 1}}{p - \sqrt{p^2 - 1}} \right], \; (p > 1)$$

$$\alpha = \frac{1}{2(1 - p^2)} \left[-p^2 - 2 + \frac{3p}{\sqrt{1 - p^2}} \text{ Arc tg} \frac{\sqrt{-p^2}}{p} \right] (p < 1)$$

IV. NUMERIC TABLES.

Numerical Tables of the functions $t(p)$, $r(p)$ and $\Lambda(p)$

(*a*) $p < 1$			(*b*) $p > 1$			
p	$t(p)$	$r(p)$	p	$t(p)$	$r(p)$	$\Lambda(p)$
1	1·000	1·000	1	1·000	1	2·50
0·9	0·997	1·015	1·5	0·985	0·838	2·63
0·8	0·996	1·0156	2	0·958	0·664	2·91
0·7	0·989	0·998	3	0·900	0·428	3·68
0·6	0·977	0·996	4	0·846	0·294	4·66
0·5	0·960	0·883	5	0·802	0·216	5·81
0·4	0·932	0·776	6	0·761	0·169	7·10
0·3	0·889	0·530	8	0·698	0·106	10·10
0·2	0·817	0·444	10	0·648	0·0748	13·63
0·1	0·686	0·232	12	0·607	0·0608	17·76
0·05	0·561	0·117	15	0·559	0·0465	24·8
0·02	0·421	0·0471	20	0·501	0·0239	38·6
0·01	0·336	0·0235	25	0·458	0·0163	55·2
0·005	0·268	0·0118	30	0·424	0·0119	74·5
0·002	0·198	0·00471	40	0·375	0·00741	120·8
0·001	0·157	0·00235	50	0·339	0·00493	176·5
			60	0·312	0·00357	242·0
			80	0·273	0·00214	400·0
			100	0·246	0·00144	593·0
			125	0·221	0·000961	610
			150	0·202	0·000694	1,222
			200	0·175	0·000412	2,051
			300	0·143	0·000196	4,278
			400	0·123	0·000116	7,247
			500	0·109	0·0000765	10,921
			600	0·0997	0·0000549	15,216
			700	0·0918	0·0000412	20,250
			800	0·0856	0·0000322	25,880
			900	0·0804	0·0000259	32,200
			1000	0·0760	0·0000213	39,117

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9

TRANSPORT PROCESSES AND ELECTRICAL PHENOMENA IN IONIC MEMBRANES

Torsten Teorell

THE mechanisms and laws governing the transport of molecules and ions in living cell and tissue membranes appear to have received an intensified interest in recent years, judging from the increased number of theoretical and experimental papers published in this particular field. The reason for this may be the fact that experimental biologists have employed more quantitative methods of research than previously and thereby accumulated quantities of numerical data suitable for theoretical approaches. One stimulating factor has been the introduction of many new and convenient methods and research tools provided by the fundamental sciences, for instance, the radioactive tracer technique. Another reason is that considerable progress has recently been made in purely theoretical studies. Many classical 'laws' and formulas have been subject to new and thorough experimental tests leading, in many cases, to extensions, improvements and completely new theories.

The following paper is intended to be a modest attempt to describe and analyse the present status and recent progress of the *physico-chemical* aspects of biological 'membrane permeability.' Certain restrictions will, however, be imposed. Only work with more or less direct biological implications will be considered. Furthermore, the 'active transport' behaviour of membranes, involving chemical energy exchange, will be omitted, although such types of transport processes have a central position in the biological research of today. It appears that there is, as yet, no sufficiently exact knowledge to permit more than qualitative descriptions, or unsupported hypotheses, of the intrinsic mechanisms involved in the alleged 'active' membrane processes.

Particular emphasis will be placed upon the permeability properties of electrolytes in a special type of membrane, namely, '*electrically charged membranes*', perhaps better called '*ionic membranes*' (a term suggested by DEAN[1], p. 516). In the writer's opinion, these membranes may come closer to the properties of the real biological cell and tissue boundaries than the neutral 'sieve' membranes generally considered in the literature of the past. The matrix of the cell membranes, blood capillary walls *etc.* are

PIB III—20

complex compounds of many constituents *e.g.* proteins, lipoids *etc.* which have an intrinsic electrolyte character in their own right *i.e.* contain *fixed ions* (compare review articles *e.g.* by DANIELLI and DAVSON[2] [Chapt. VI] or DAVSON[3] [Chapt. VIII]). The amphoteric proteins, for instance, have immobile or 'fixed' $-COO^-$ and $-H_3N^+$ groups, which are capable of binding cations or anions respectively as '*gegen*' ions. These are exchangeable with ions in the surroundings (*i.e.* cytoplasma, interstitial fluid or the blood plasma). Dissociating groups of other membrane constituents may also contribute to the 'fixed charge' density. Furthermore, there exist oriented polar groups, which can probably exert additional electrical effects. It may therefore, be an oversimplification, in most cases, to regard living membranes as porous, electrically neutral sieves. It should be emphasized that another permeability concept, which has been used to explain much of the transport behaviour of organic molecules, namely, that of 'oil' or 'solubility' membranes, is not different in its fundamental nature from that of dense, homogeneous 'fixed charge' membranes, a fact pointed out already by WILBRANDT[4,5].

RECENT SUMMARIZING LITERATURE

The recent progress pertaining to membrane diffusion and transport problems has been achieved in many varying fields of research, in which the starting points and aims have been different. Papers in physical chemistry, technology as well as in animal or plant physiology and soil science, contain contributions of interest, as can be observed from the references of this paper. A complete follow up of the recent literature has therefore not been possible within the scope of this article. There are, however, a number of summarizing papers and books published to which the reader may be referred for more complete references and, in particular, for information on fundamental concepts.

DEAN'S[1] excellent review of 1947 on 'The Effects produced by Diffusion in Aqueous Systems containing membranes' contains, besides a host of references, constructive discussions of the problems involved. Another valuable article dealing with the electrochemistry of 'charged membranes' is that of SOLLNER[6]. The more biological aspects of membrane permeability have been treated in some recent books : DAVSON'S[3] *General Physiology* summarizes and extends certain parts contained in the somewhat older but still stimulating book by DAVSON and DANIELLI[2] on *Permeability of Natural Membranes*. The book *Electrical Phenomena at Interfaces* (edited by BUTLER[7]) contains many interesting chapters, and FLOYD'S contributions (Chapt. X and XI) on 'Membrane Potentials and Electrical Properties of Living Cells' and on 'Electrical Properties of Nerve and Muscle'

are especially worthy of mention. CRANE[8] has thoroughly treated 'Bioelectric Potentials, their Maintenance and Functions' in Volume I of this series (with 279 references). HODGKIN[55] has recently written an admirable account of the ionic basis of electrical activity in nerve and muscle. These publications relieve the present writer of the necessity of reviewing the abundant biological literature on electrical phenomena. Two recent review articles on 'Permeability' by TEORELL[9] and by STEINBACH[10] may also be mentioned; the former attempts some critical discussion on the present position of permeability research.

As outlined above, this article will take a particular interest in 'charged' or 'ionic' membranes. Such membranes have the properties of 'ion exchangers,' and the mass of information available on artificial and natural ion exchangers is therefore of great importance. Comprehensive monographs dealing with the artificial ion exchange resins have been written by NACHOD[11]; by KUNIN and MYERS[12], and also by MYERS[13] and BOYD[161]. Many problems of chromatography are similar to those of membrane equilibria : a survey of this type of adsorption analysis has been published by STRAIN[14] and many important papers can be found in the *Faraday Society Discussion* 1949 (No. 7) on 'Chromatographic Analysis'[15], several of which will be excerpted below.

More remote subjects, which have implications on the membrane transport kinetics, have been treated by TAYLOR[16] and by BARRER[17]; a somewhat older collection of papers, which deserve to be consulted, are those discussed under the title 'The Electrical Double Layer'[18].

A GENERAL CHARACTERIZATION OF PERMEABILITY PHENOMENA IN MEMBRANES

The biological term 'permeability', taken in a narrow sense, is a quantitative measure of molecular or ionic transport, but it is often used in a somewhat wider and ambiguous way to include other phenomena related to the ionic transport, such as membrane potentials, electrical conductivity *etc.* A survey of such interrelated phenomena is given in *Table I.* A complete and satisfactory theory of 'passive' membrane permeability ought to be able to describe all these phenomena in terms of the physico-chemical properties of the membrane and the nature and composition of the surrounding solutions. An approach to such a theory was recently published by the reviewer, TEORELL[19]; although provisional and incomplete in many respects, it will be used in the following as a 'skeleton' for a wider discussion.

Table I. Various ionic processes in membrane systems, 'Permeability phenomena.'

1. *Ionic transport, "flux".*
   Net flux, partial fluxes of different ion species.
   'Influx' and 'outflux' of one ion species.
   Flux ratios.
2. *Membrane potential.*
   Potential components, 'mixed' or total potential.
   The diffusion potential.
3. *Electrical conductivity.*
   Conductances in steady state systems.
   Membrane rectification.
   Reactance properties, apparent capacitance and inductance.
4. *Ionic distribution equilibria.*
   'Flux equilibria', 'diffusion effects'.
5. *Spatial distribution of the ions and the potential within the membrane.*

Some fundamental definitions of driving forces and transport

The ambiguity of the term permeability, as often used, can be traced back to the fact that many authors in the past have not made a clear distinction between (*a*) the '*permittivity*' of a membrane for a particular substance under investigation *i.e.* the permeability in the proper, narrow sense (this is usually described as 'mobility' *i.e.* the velocity of the substance under the unit force) and (*b*) the *driving forces*, which create the very process of transport (the permeation or penetration). It has not always been understood that any such transport must arise from the simultaneous operation of both of the factors (*a*) and (*b*); the presence of either of them alone does not lead to any penetration. Further comments on 'false or apparent impermeability' and on the distinctions between permeability proper and the nature of superposition of driving forces can be found in a previous paper by the author (TEORELL[9], pp. 546-551).

In order to avoid the possible ambiguity of the word permeability when dealing with transport one may exchange it for 'flux'. In *any* part of a membrane system, the flux is defined as :

$$Flux = mobility \times concentration \times total\ driving\ force \quad \ldots . (1)$$

Here *flux* = *amount* of substance which per time unit penetrates per unit area normal to the direction of the transport. In equation (1) all factors refer to a given volume element and the force is defined per unit of concentration (moles or gram ions).

The intensity of the flux of a substance referred to unit conditions is often termed the *diffusion coefficient* or the *permeability constant*, having the dimensions $cm^2 sec.^{-1}$. A clear discussion on the definitions and dimensions of the diffusion coefficients *etc.* can be found in DEAN'S[1] paper.

It should be emphasized that the total force can be composed not only of concentration (activity) gradients, but may also include superimposed forces originating from an electrical potential, hydrostatic-osmotic pressure

gradient *etc.* (This is actually the case in ionic membranes, as will be evident below). *Table II* shows how different constituents in an aqueous membrane phase can be directly influenced by the commonly existing forces. Figure 1 is a scheme visualizing the forces acting upon a single

Table II. Common driving forces acting in a membrane phase and the type of particles influenced thereof

Force		
'*Osmotic*'	'*Electric*'	'*Hydrostatic*'
Concentration (activity) gradient (= chemical potential gradient)	Electrical potential gradient	Pressure difference (osmotic ± hydrostatic)
electrochemical potential gradient		
$RT\frac{d(\ln c^{+\prime})}{dx}$ $\left(=\frac{RT}{c^{+\prime}}\cdot\frac{(dc^{+\prime})}{dx}\right)$ §	$zF\cdot\frac{d\varphi}{dx}$	Can be expressed in terms of a linear flow velocity μ of the solvent
$\frac{d\mu^{+\prime}}{dx}$		
Molecules Ions (Colloids)* —	— Ions (Colloids)† (charged suspensions cells, *etc.*)†	Molecules Ions (Colloids)† (charged suspensions)†

* Generally negligible.
† Acting only if membrane allows permeation of the particles.
§ Activity is taken equal to concentration.

cation species in a membrane (free from convections and hydrostatic flow): The concentration gradient in the chosen membrane section is $dc^{+\prime}/dx$ (negative) and the 'osmotic' force (per gram ion) in the positive direction (1) to (2) is $-(RT/c^{+\prime}).dc^{+\prime}/dx$; the electrical potential gradient is $d\varphi/dx$ positive and the electric force on one gram ion of cations is $-z\,F\cdot d\varphi/dx$ (the significance of symbols is given on p. 319). The *total* force obtained by superposition is, according to the well known NERNST-PLANCK principle, their algebraic sum, equal to

$$\textit{total driving force}\ (\text{per gram ion}) = -\left(\frac{RT}{c}\frac{dc}{dx}+zF\frac{d\varphi}{dx}\right) \quad \ldots . \quad (2)$$

One should note that, in the figure the gradients dc/dx and $d\varphi/dx$ have opposite signs *i.e.* the electric force here counteracts the osmotic force and hence tends to cause a *retardation* of the movement of the *cations* in question. For an *anion* (where the valency z should be taken as negative) an *acceleration* would be obtained. A further illustration of the co-operation

of the 'osmotic' and 'electrical' force resulting in unequal ion distributions is depicted in Figure 13.

The modifying influence of a hydrostatic flow which can exert an extra driving force will be discussed later (p. 363).

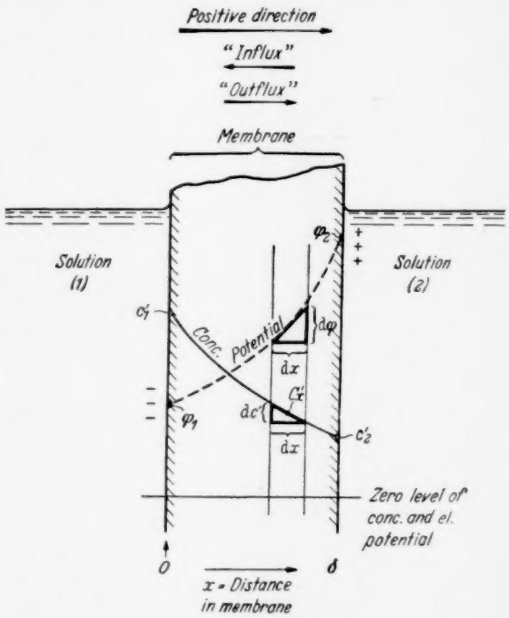

Figure 1. Scheme of the forces acting on a cation species within a membrane. A force is reckoned positive when it tends to cause a transport in the direction (1) → (2). The *osmotic* force being $-(RT/c)dc'/dx$ is here positive (because dc'/dx is negative). The electric force, $-zF \cdot d\varphi/dx$ is here negative, hence causing a decrease in the total driving force, resulting in a retarded cation transport.

The fundamental flux equation (1) is quite *general* and valid for any system where material transport is possible *i.e.* for simple diffusion in 'open' systems without hetereogenities, as well as for systems with different phases such as those prevailing in biological organisms. It is also applicable when an electric current flows.

SOME MODERN CONCEPTS ON TRANSPORT PROCESSES IN MEMBRANES

The elementary part of a biological transport system is a 'membrane' with surrounding aqueous 'bulk' solutions of molecules, ions and dispersed particles. In attempts to gain insight into the events within the biological membranes, a number of 'models' have been proposed, which have been subjected to experimental and theoretical studies. The type by far the most frequently employed have been inanimate membranes of a porous nature such as sheaths of collodion or cellophane, sintered glass *etc.* surrounded by stirred solutions.

Deviations from the simplest membrane process

Development of new theories—Usually one has regarded the *simplest* membrane transport process as similar to diffusion in a confined watery phase, which is 'convection proof'. Furthermore it has been tacitly assumed (*a*) that the external membrane surfaces are in a permanent, constant

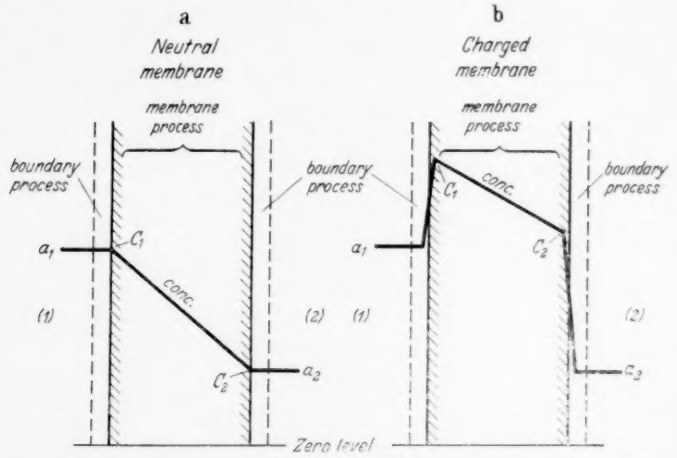

Figure 2. Scheme of the variation of the concentration of an ion species at a 'neutral' membrane (*a*) and at a 'charged' membrane (*b*). Note that in spite of identical bulk concentration *difference* (a_1—a_2), the concentration *gradient* within the membrane is smaller in the case (*b*).

equilibrium with the external solutions, and (*b*) that the solute concentrations in the 'openings' and 'ends' of the membrane pores can be taken as equal to those in the respective outside solutions (see Figure 2*a*) :

A picture resembling Figure 2*a* has been employed in theories like FICK's classical 'laws' for molecular diffusion and is inherent in the well-known expressions of NERNST-PLANCK and others for electrolyte *diffusion potentials*. Experiments on *coarse*, porous membranes have generally shown a reasonable agreement with the theories mentioned. In a great many other cases, however, various types of deviations have been reported, particularly in biological objects. A common observation has been that the penetration of molecules and many ions was very appreciably slowed down in certain membranes, more than could be explained by simple 'sieve effects' or restrictions of the available diffusion area. The membranes in question have generally been more like 'oil' membranes than a pore-water system. Another experience has been that animate as well as many inanimate membranes have exhibited a 'selective ion permeability' for either cations or anions, generally measured with the aid of the 'membrane potential'. A much discussed anomalous result of the latter type is known as the 'concentration effect' (compare p. 331).

These 'deviating' cases of penetration have recently led to the development of two different lines of a theoretical approach to membrane transport problems. One may be called the *transition* (= activated) *state theory* and is especially adapted for the non-aqueous membranes. The other has been named the *fixed charge theory* and aims at an explanation of the selectivity of the ionic transport and the electrogenesis in membranes. Both focus the attention on the conditions at the solution-membrane interfaces (the *boundary process*) as well as at the internal *membrane process*. The distinction between the 'classical' membrane concept and the recent ones is schematically shown in Figure 2*a* and 2*b* respectively. In contrast to the simplest case with identical concentrations (and electrical conditions) in bulk and in the adjoining membrane interfaces, the new theories anticipate *unequal concentrations across the phase boundaries*. The transition state theory explains the concentration jumps as due to different 'rate processes' leading to different 'partition', while the fixed charge theory operates with an electrochemical ion distribution mechanism (the DONNAN equilibrium). Owing to the discontinuity of phase at the membrane surfaces, one has to consider the possibility that either the boundary processes or the membrane process may become the rate controlling factor. The transition state concepts include both possibilities, while the fixed charge formulas only consider the kinetics of the interior membrane process as rate determining, because the boundary process is assumed to be very fast.

Both theories have their specific advantages and disadvantages. The transition state concept provides a general, unified view applicable to systems of varying degrees of complexity. It operates only with energetic or thermodynamical quantities. Therefore, it is difficult to subject it to experimental tests without access to such quantities. The fixed charge theory, on the other hand, is less abstract in its nature, at least for biologists. It rests on the classical diffusion concepts of NERNST-PLANCK, the well known equilibrium ideas of DONNAN and is primarily applicable only to aqueous systems. From the experimental point of view it is easy to use, because only concentration and mobility quantities enter, which are determinable in one way or another.

It should be emphasized that the free energy concept, as exposed in the transition state theory, is in no way contrary to the purely kinetic views employed in the fixed charge theory. Both theories apply to a heterogeneous three layer system, although they use different 'languages'. In fact, they yield in comparable cases identical results. It remains, however, to work out their mutual relationships more in detail. Although the transition state theory in its present state of development does not expressively take into account the effect of a charged or ionic membrane, we think that a short sketch of its basic views will be instructive. We will

also use some of its results, later on, in a discussion on the short-comings of some assumptions underlying the fixed charge theory (compare p. 360).

An outline of the 'transition state' theory

A thorough treatise of membrane diffusion based on the 'activated state' or 'transition state' theory was recently published by ZWOLINSKI, EYRING and REESE[20]. The first considerations along these lines were, however,

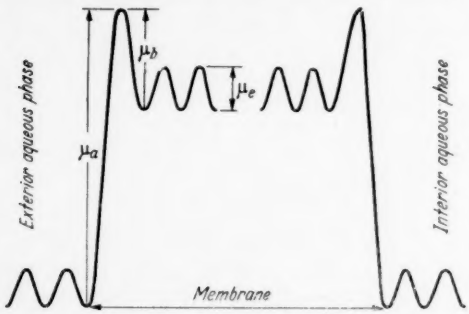

Figure 3. Energy profile across a cell membrane (according to DANIELLI). For explanations see text.

published by DANIELLI[2] in 1943 (Chapter XXI and Appendix A). For the sake of a later discussion we prefer here to follow DANIELLI'S analysis of the significance of the 'permeability constant'. We quote from an excellent digest by DAVSON[3]:

> 'The process of penetration can thus be represented by a simple potential energy diagram as in Figure 3; the molecule to enter the membrane, must make a potential energy-jump, of height μ_a (*i.e.*, it must wait until it gets this amount of energy before making the transition). Within the membrane it has a smaller potential energy than that necessary to enter, *i.e.*, it tends to adhere to the membrane molecules, and, to pass back into the aqueous phase, it must detach itself from these *i.e.* make the jump μ_b. In passing through the membrane, a succession of small jumps are made of height μ_e; on leaving the membrane a jump of height μ_b must be made, μ_b being a measure of the attraction of the membrane molecules for the substance considered. The actual rate of penetration is therefore a function of a number of energy-jumps, and if these can be determined, it should be possible to calculate the permeability constant. It should be noted that the potential jumps μ_a and μ_b determine the partition coefficient, the relative concentrations of a molecule in two layers of fat and oil being determined by the ease with which the molecules can pass from one layer to the other; in fact the partition coefficient is equal to the ratio a/b, where a measures the rate of passage through the potential barrier μ_a, and b that through the barrier μ_b. According to DANIELLI'S mathematical analysis, the permeability constant, P, is given by:
>
> $$P = \frac{ae}{nb + 2e} \qquad \ldots . (3)$$
>
> where a, b, and e are rates of diffusion across the potential energy jumps μ_a, μ_b, μ_e, and n is the average number of potential energy jumps within the membrane. This analysis is of great value in that it enables us to see under what conditions the partition coefficient will determine the rate of penetration, and also the mathematical form of the relationship between permeability constant and partition coefficient.'

The recent new treatment of ZWOLINSKI, EYRING and REESE[20] is essentially similar : They operate with four rate constants for diffusion in their energy profile curve : k_s (in solution), k_m (in membrane), k_{sm} (through solution-membrane) and k_{ms} (for membrane-solution). The distribution or partition coefficient between solution-membrane is defined as the ratio $K = k_{sm}/k_{ms}$. Depending on the values of the k's, various cases can be distinguished. When dealing with natural membranes surrounded by aqueous solutions, they assume the distribution coefficient for most non-electrolytes to be much less than unity ($k_{sm} \ll k_{ms}$). With this condition three cases are considered :

$$\begin{aligned} &\text{I.} && k_m \ll k_{sm} \ll k_{ms} \\ &\text{II.} && k_m \gg k_{ms} \gg k_{sm} \\ &\text{III.} && k_m \approx k_{ms} \approx k_{sm} \end{aligned}$$

It is interesting that the authors, by analysing data in the literature on the permeability of various biological membranes (CHARA, ARBACIA *etc.*) have found that all three classes are represented (a somewhat similar analysis was previously done by DANIELLI). These authors also obtain expressions for the permeability constant which are similar to DANIELLI's constant P in terms of the above-mentioned rate symbols.

Some important remarks have to be made here as to the significance of the permeability constant P as used by DANIELLI and the EYRING group. The flux, ϕ, the mobility u and the *concentration difference* between the surrounding solutions, $(c_2' - c_1')$, are related as $\phi = -P \cdot u\,(c_2' - c_1')$. In the fixed charge theory, as will be demonstrated later on, there appears also a kind of permeability constant K, which is, however, related to the *electrochemical activity difference*, whereby the flux is defined as $\phi = K \cdot u\,(c_2'\xi - c_1')$. Here ξ is a factor which takes into account the electric potential. Accordingly it is important not to confuse the different 'permeability measures' P, u and K.

LAIDLER and SHULER[21,22] have also treated the kinetics of membrane transport under steady state conditions. They employ similar principles and express the rate constant of the over-all process of surface penetration in terms of three specific rate constants. Various special cases are considered and discussed with reference to the experimental data. They develop flux equations for solvent and solute, especially as a function of the osmotic and hydrostatic pressures across the membrane. In a further paper SHULER, DAMES and LAIDLER[23] tested their expressions on the diffusion of various non-electrolytes and of water and also calculated the 'energy of activation' and 'entropy of activation'. The former was found to be only slightly less than for free diffusion (about 2 to 5 kcal).

The 'transition state theory' of DANIELLI-EYRING *et al.* has certainly large potentialities of further extension and has an intrinsic beauty arising

from its unified and fundamental character. In its present state of development, however, it does not provide a basis for explanations of other permeability phenomena as the electric membrane potential, membrane conductivity *etc.* listed in *Table I*. These phenomena as well as the flux process can be coped with by aid of a different mode of approach *i.e.* the 'fixed charge theory' which will be outlined as to its principles in the next section.

The original fixed charge theory

1. Already in the early epochs of permeability research one spoke of cation permeable ('negative') or anion permeable ('positive') membranes. In particular, MICHAELIS and his collaborators in the twenties and early thirties characterized the permeability of dense membranes (dried collodion *etc.*) in terms of the electrical potential measured in a solution-membrane-solution system. The potentials measured deviated markedly from those required by the NERNST-PLANCK equations for unrestrained diffusion (compare 'the concentration effect' mentioned on p. 311). A first step towards a rational explanation of the behaviour of this type of membrane was taken by WILBRANDT[1], who pointed out the similarity between the two phase boundary potentials at 'oil' membranes (according to BEUTNER and others) and the interfacial potentials at the dried collodion membranes, to which he ascribed a quasi-crystalline structure. Assuming complete exclusive cation permeability, he calculated the sum of these opposing interface potentials according to a mass law concept and assumed that this summed potential greatly exceeded the diffusion potential due to the gradient in the pores. In connection with work on the apparent ionic transference number in cellophane membranes (TEORELL[24]), the author in 1935 published an attempt to formulate a theory of the permeability of 'charged' membranes of the MICHAELIS-WILBRANDT type (TEORELL[25,26]). The main features of this theory were:

(*a*) The membrane itself is regarded as having a charge due either to 'adsorption', 'dissociation', or 'polar character' *etc.* but it is not necessary to make any further assumption as to its nature. The effect of the membrane is regarded as that of an 'added ion' of a fixed, homogeneous concentration. All ions were regarded as permeable through the membrane with given mobilities (not necessarily different from those in free water).

(*b*) There exist permanent DONNAN equilibria between the external solutions and the membrane surfaces.

With these assumptions it was possible to give an expression for the *total membrane potential* as equal to the sum of (*a*) two DONNAN potentials at the membrane boundaries and (*b*) a common diffusion potential within the membrane (which for simplicity was always calculated from the

HENDERSON formula). By aid of the new formula one could explain the 'concentration effect' quite satisfactorily.

Independently, in 1936, an identical theory was published at a greater length by MEYER and SIEVERS[27-30,31], who also, in a number of later papers,

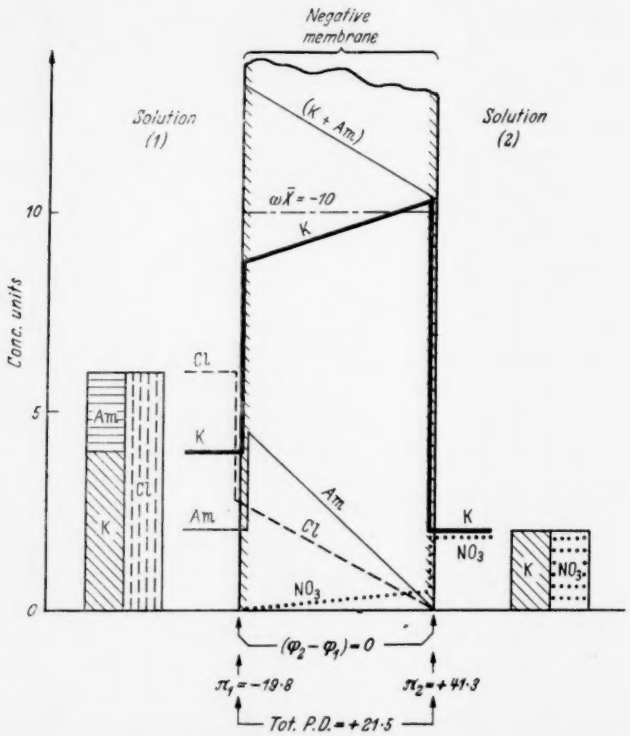

Figure 4. The variation of the ion concentrations at a charged membrane where the interior membrane potential is zero (compare Figure 6). An actual counterpart would be a system as depicted with the arrangement: (1) 4 mN KCl + 2 mN NH_4Cl ‖ membrane $\omega X = -10$ mN ‖ 2 mN KNO_3 (2). The ionic mobilities of K^+, NH_4^+, Cl^- and NO_3^- assumed to be identical. In this case the respective concentration gradients inside the membrane signify direction and magnitude of the ion fluxes. Note that the K^+ ions flow in the direction (2) → (1) *i.e.* from a lower to a higher concentration. A similar situation would arise with any electrolyte mixture when the 'membrane concentration' is high, relative to the bulk total concentration (see text).

extended the applicability to liquid membranes and tested the theory in numerous experiments.

It should be pointed out explicitly, that the 'fixed charge' membrane theory in the original publications of TEORELL-MEYER-SIEVERS only aimed at describing the *membrane potential* in terms of ion concentrations, ion

mobilities and 'membrane charge'. Nevertheless a great many applications of it have been published in the last decade.

2. An extension of the original theories to include also the transport processes *i.e.* the partial *ion fluxes* and the *conductivity* and expressions on *ionic distribution effects* has been highly desirable. Such an extended theory has recently been presented by the author (TEORELL[19]) and will be considered at some length below.

An *approximate* method of approach to the *diffusion kinetics* is inherent in the original presentation. In order to get an explicit flux equation from the general flux formula (Eq. 1), one requires knowledge of the steady state distributions of the concentrations of the mobile ion *within* the membrane as well as information of the course of the interior membrane potential. As regards the latter, one finds, by examining the TEORELL-MEYER-SIEVERS formulas, that it tends to vanish when the ratio ('membrane charge') : (external total concentration) is *high* (of the order of 10). For such a *special case* one would expect all concentration gradients in the membrane to become approximately linear, see Figure 4. Hence FICK's elementary diffusion law could be applied to an ion species as (u' is mobility, δ is thickness)

$$\text{net flux per unit area} = -\frac{RT}{\delta} \cdot u' \cdot (c'_2 - c'_1) \qquad \ldots . (4)$$

Here the c'_1 and c'_2 denote the partial ion concentrations (activity) at the *membrane surface* (1) and (2). These are in turn equal to $a'_1 r_1$ and $a'_2 r_2$, where a'_1 and a'_2 are the partial concentrations of the external solutions and r_1 and r_2 the respective DONNAN equilibrium ratios, which can easily be calculated when the ratios (membrane charge) : (external total concentration) are known. This simplified method constitutes actually the essence of some calculations on ion fluxes recently performed by ERIKSSON[32], who, however, rightly admits that the method 'may look very rough'

3. *Shortcomings of the original fixed charge theory*—It can be said that the main objection to using the original DONNAN-HENDERSON ion distribution concept resides in the fact that the HENDERSON method requires 'linear mixtures' in every section of the membrane. The simplest such mixtures would be obtained when all concentration gradients are linear. Such a state is, however, contrary to the requirements for a steady state in a convection-free, constrained boundary as pointed out by PLANCK[33] and PLETTIG[34]. They showed theoretically that the *concentration gradients are non-linear*, and it could even be expected that the concentration distribution in the diffusion layer exhibited *maxima*. This prediction has been confirmed in some experiments of TEORELL[35,19], one of which is presented in Figure 16. Compare also further discussions on p. 355.

Accordingly, the assumption of a HENDERSON distribution, as used by TEORELL-MEYER-SIEVERS, cannot be correct in the *general* case of external solutions of electrolyte *mixtures*. A correct treatment would require the introduction of PLANCK's concepts instead of HENDERSON's. Such a revision has recently been made by TEORELL[19]. It necessitates a more elaborate mathematical procedure, but it is certainly worth while, because

it is possible thereby to obtain exact expressions also for ion fluxes *etc.* It should be pointed out, however, that the HENDERSON and PLANCK methods yield identical partial results (as regards the interior *potential*) in two important *special* cases : (*a*) when only one single 1:1 valent electrolyte diffuses, and (*b*) when the *total* concentrations in the two external solutions are equal (compare p. 328 and 329).

AN EXTENDED 'FIXED CHARGE' THEORY FOR PERMEABILITY PROCESSES IN IONIC MEMBRANES

Ionic transport

The results of a renewed and extended theory yielding expressions of general validity for membrane ion fluxes as well as for the accompanying

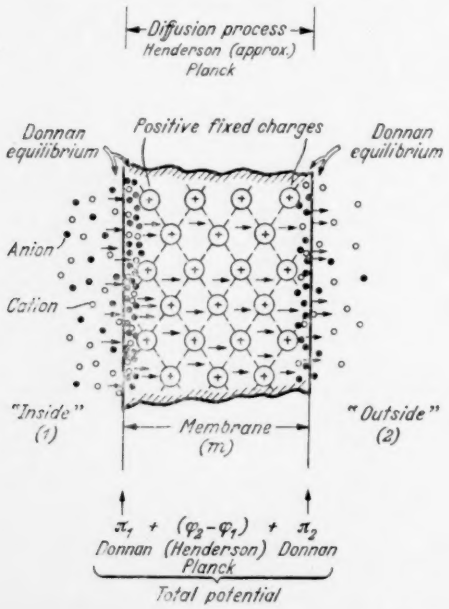

Figure 5. Diagram illustrating the basic concepts of the fixed charge theory.

potential, conductivity *etc.* will now be presented following the paper by TEORELL[19].

A scheme illustrating the assumed conditions of the 'fixed charge' theory is given in Figure 5.

1. *The basic assumptions* are:

(*a*) The membrane matrix is rigid, of constant volume and behaves as a (strongly) ionized exchange body with fixed ions or 'charges' of a constant volume density referred to the aqueous interstices (called '***membrane concentration***').

(*b*) The membrane interstices are permeable to *all* ions in the external solutions and are homogeneous throughout the membrane. All ions are 1:1 valent.

(*c*) Osmotic or hydrostatic flow of water is negligible.

(*d*) The membrane surfaces are in a state of permanent, *instantaneously* established DONNAN equilibrium with the same distribution ratio for all cations (and the inverse value valid for all anions). This assumption implies that the membrane process entirely controls the rate of ion fluxes.

The validity of these assumptions will be discussed later on p. 356.

2. *Notations*

ϕ = net flux, *i.e.* the *amount* of an ion species passing unit area of membrane in the time unit.

$u', u'', \ldots v', v'' \ldots$ = absolute, constant mobilities of cations resp. anions *within* the membrane.

RT = gas constant $\times$ absolute temperature.

c = ion concentration in the membrane.

a = ion concentration in external solution.

c^+, c^-, a^+, a^- refer to cations or anions; dashed symbols c', $c'' \ldots$ refer to single ion species; not dashed denote total concentration ($c = c' + c'' + \ldots$).

r = the DONNAN distribution ratio, compare Eq. (8).

$\omega \bar{X}$ = the constant 'membrane charge' or 'membrane concentration' of fixed ions (in the same unit as the c:s). The ω is + 1 for cations and − 1 for anions.

x = the distance in membrane, extending from $x = 0$ at the interface (1) to $x = \delta$ at the other interface (2).

φ = the electrical potential within the membrane (usually taken $\varphi_1 = 0$ at $x = 0$).

F = the *Faraday* constant.

z = valency.

$\beta = F/RT$.

$\xi = \exp(F\varphi/RT)$.

Indices (1) and (2) refer to the two external solutions, or membrane interfaces.

Further abbreviations will be explained in the text.

3. *The fundamental ion flux equation*

The Eqs. (1) and (2) according to NERNST-PLANCK can be transformed as follows (for a *cation* species) if one employs PLANCK's convenient auxiliary expression $\xi = \exp(F\varphi/RT)$ or $\ln \xi = (F\varphi/RT)$:

ion net flux = *mobility* . *concentration* . *total force* (per gram ion).

$$\textit{cation net flux, } \phi^{+\prime} = -u^{\prime} \cdot c^{+\prime} \cdot \left(\frac{RT}{c^{+\prime}} \frac{dc^{+\prime}}{dx} + F \frac{d\varphi}{dx} \right) \quad \ldots . \text{(1:1)}$$

$$= -RT \cdot u^{\prime} \cdot c^{+\prime} \left[\frac{d\,(\ln c^{+\prime})}{dx} + \frac{d\,(\ln \xi)}{dx} \right] \quad \text{(1:2)}$$

$$= -RT \cdot u^{\prime} \cdot c^{+\prime} \cdot \frac{d\,(\ln c^{+\prime} \xi)}{dx} \quad \ldots . \text{(1:3)}$$

Similarly for

$$\textit{anion net flux, } \phi^{-\prime} = -RT \cdot v^{\prime} \cdot c^{-\prime} \frac{d\,(\ln c^{-\prime}/\xi)}{dx} \quad \ldots . \text{(1:4)}$$

The integration of Eq. 1:3 for a *steady state* condition, remembering that $c^{+} - c^{-} + \omega \bar{X} = 0$ (electroneutrality), yields the equation (7) (for a *cation*).

Before writing down the result, the following abbreviations are defined. K^{+} is a factor *common* to all cation species given by

$$K^{+} = \frac{RT}{\delta} \cdot \left\{ \frac{(c_2^{+} - c_1^{+}) - 0{\cdot}5 \cdot \omega \bar{X} \cdot \ln \xi}{(c_2^{+} \xi - c_1^{+})} \cdot \frac{\ln \bar{k} \xi}{\ln \bar{k}} \right\} \quad \ldots . \text{(5)}$$

and

$$\bar{k} = \frac{c_2^{+} + 0{\cdot}5\,(\ln \bar{k}\xi)/(\ln \bar{k}) \cdot \omega \bar{X}}{c_1^{+} + 0{\cdot}5\,(\ln \bar{k}\xi)/(\ln \bar{k}) \cdot \omega \bar{X}} \quad \ldots . \text{(6:1.2)}$$

$$\left(\text{or } \frac{c_2^{-} - 0{\cdot}5\,(\ln \bar{k}/\xi)/(\ln \bar{k}) \cdot \omega \bar{X}}{c_1^{-} - 0{\cdot}5\,(\ln \bar{k}/\xi)/(\ln \bar{k}) \cdot \omega \bar{X}} \right)$$

The *general flux equation* (for a cation) is then

$$\underline{\phi^{+\prime} = -K^{+} \cdot u^{\prime} \cdot (c_2^{+\prime} \xi - c_1^{+\prime})} \quad \ldots . \text{(7)}$$

Introduce now the assumption that the membrane interface concentrations (c) and the bulk concentrations (a) are related according to the DONNAN principle with the ion distribution ratio

$$r = \frac{c^{+\prime}}{a^{+\prime}} = \frac{c^{+\prime\prime}}{a^{+\prime\prime}} = \cdots = \frac{c^{+}}{a^{+}} = \frac{a^{-\prime}}{c^{-\prime}} = \frac{a^{-\prime\prime}}{c^{-\prime\prime}} = \cdots = \frac{a^{-}}{c^{-}} \quad \text{(8:1)}$$

where r can be calculated from

$$r = \sqrt{1 + \left(\frac{\omega \bar{X}}{2a} \right)^2} - \left(\frac{\omega \bar{X}}{2a} \right) \quad \ldots . \text{(8:2)}$$

One can now rewrite (7) in terms of external concentrations

$$\underline{\phi^{+\prime} = -K^{+} \cdot u^{\prime} \cdot (a_2^{+\prime} \cdot r_2 \xi - a_1^{+\prime} \cdot r_1)} \quad \ldots . \text{(9)}$$

Corresponding expressions for *anion* species are formed by exchanging c^+ for c^-, a^+ for a^-, r for $1/r$, u' for v', K^+ for K^-, ξ for $1/\xi$ with exception of the term $(0{\cdot}5\ \omega\bar{X}\cdot\ln\ \xi)$ in (5), and the constant k remains also unchanged.

The significance of (7) can be expressed : *the flux of an ion species inside the membrane is proportional to its mobility and to the difference in electrochemical activity* between the membrane interfaces. The proportionality constant $K^{\pm}$ is, however, dependent on both the total concentrations of the mobile ions and the membrane charge, according to (5) and (6).

The term *electrochemical activity* needs some elucidation : in analogy with BRÖNSTED-GUGGENHEIM[36] one can define the electrochemical potential for any *cation*, as $\bar{\mu}^{+\prime} = \bar{\mu}_0{}^{+\prime}$ (standard state) $+ RT(\ln\ \bar{a}^{+\prime})$, which differentiated becomes $d\bar{\mu}^{+\prime}/dx = RT\cdot d(\ln\ \bar{a}^{+\prime})/dx$. The electrochemical potential can be regarded as the sum of the chemical and electrical potential, whose gradients $(RT/c^{+\prime})\cdot(dc^{+\prime}/dx)$ and $d\varphi/dx$ entered in the differential flux equation (1:1). This expression could now be rewritten

$$\phi^{+\prime} = -\ u'\cdot c^{+\prime}\cdot\frac{d\bar{\mu}^{+\prime}}{dx} \qquad \ldots\ (10{:}1)$$

$$= -\ RT\cdot u'\cdot c^{+\prime}\cdot\frac{d(\ln\ \bar{a}^{+\prime})}{dx} \qquad \ldots\ (10{:}2)$$

By comparing (1:3) and (10:2) it becomes obvious that $\bar{a}^{+\prime} = (c^{+\prime}\xi)$, hence $(c^{+\prime}\xi)$ has the form of an 'electrochemical activity'. Incidentally it should be remarked that GUGGENHEIM regards the splitting up of the electrochemical potential in a chemical and electrical part as without any physical significance, but it appears nevertheless to be a useful convention. USSING[37–39, 148] also employs the electrochemical potential concept when dealing with diffusion in uncharged membranes.

It should be observed that flux equation (7) or (9) is *perfectly general* and hence valid both in case of free diffusion (when no electric current (flows) and in case of an externally imposed electric potential (when current flows).

The *numerical evaluation of* K^+ *or* K^- is cumbersome in the general case due to the transcendental nature of (5) and (6). It must be preceded by knowledge of, or calculation of the interior membrane potential difference $(\varphi_2-\varphi_1)$ in order to obtain a value of ξ (compare the $(\varphi_2-\varphi_1)$ formulas on pp. 327, 328). The principles of the algebraic procedure are outlined in the original paper ([19], p. 469 see also p. 328).

A formula related to (7) has been derived also by GOLDMAN[40]. He did not however, pursue the problem to any definition of the relations between the concentrations in the membrane and those in the surrounding bulks.

4. *Some special cases*

(*a*) *Molecular diffusion*—Equation (7) can be regarded as a generalized form of FICK's second law for *molecular* transport, in which case $\xi = 1$ (the membrane potential and the membrane charge are of no effect here). For such a special case one also obtains the ion distribution ratio

$r = c^+/a^+ = 1$ when (7) and (9) reduces to the well-known FICK equation

$$\phi' = -\frac{RT}{\delta} u' \cdot (a_2' - a_1')$$

(*b*) *Flux in uncharged membranes containing ion mixtures*—Insertion of the value zero of the membrane charge $\omega\bar{X}$ in (5) and (6) reduces (7) to a simpler form

$$\phi^{+\prime} = -\frac{RT}{\delta} u' \cdot \frac{(a_2 - a_1)}{(a_2\xi - a_1)} \cdot \frac{\ln (a_2\xi/a_1)}{\ln (a_2/a_1)} \cdot (a_2^{+\prime}\xi - a_1^{+\prime}) \ldots . \quad (11{:}1)$$

$$= -K^+ \cdot u' \cdot (a_2^{+\prime}\xi - a_1^{+\prime}) \quad \ldots . \quad (11{:}2)$$

Equation (11:1a) was obtained already in 1897 by BEHN[41]. Also here information regarding ξ, *i.e.* of the membrane potential is necessary. In the case of free diffusion one can use PLANCK's equation (*cf.* p. 327) or the more convenient but approximate HENDERSON formula. It seems not to have been appreciated that BEHN'S equation of type (11:1) provides *an exact solution of the kinetics of diffusion in* 1-1 *valent electrolyte mixtures* (a problem that has received considerable attention particularly by MCBAIN and DAWSON[42] (compare VINOGRAD and MCBAIN[43] and DEAN[1,44])). Here, too, the flux equation can be interpreted as : 'the rate of steady state transport is proportional to the difference in electrochemical activity between the surrounding solutions'. Equations (11) as well as (9), are, valid also when external potentials are applied (when ξ refers to the potential drop inside the membrane, compare p. 328).

(*c*) *Some special cases with ionic* (*charged*) *membranes*—1. *Single salt* : A common experimental condition used by many workers on membrane permeability has been the employment of a single 1:1 valent electrolyte at a different concentration inside and outside of the membrane. In absence of electrical current flow *i.e.* with free *diffusion*, the general flux equation (7) takes the form

$$\phi^{\pm} = -\frac{RT}{\delta} \cdot \frac{2\,u\,v}{u+v} \left[(c_2^+ - c_1^+) - \frac{(\varphi_2 - \varphi_1)}{50 \cdot 4} \cdot \omega\bar{X} \right] \quad \ldots . \quad (12)$$

The *interior* membrane potential $(\varphi_2 - \varphi_1)$ can be found by the appropriate HENDERSON formula, which is applicable here (compare equations (26) on p. 328). The flux ϕ is now equal for the cation and the anion. Figure 7 (p. 329) represents a numerically worked out case for N/10 HCl diffusing against N/100 HCl across positive and negative membranes of varying charge. The ϕ curve suggests that *the flux of a single electrolyte should diminish with increasing membrane charge*. This seems to fit in with the experimental experience (WILBRANDT[4], TOLLIDAY *et al.*[155]). The penetra-

tion of neutral molecules will remain unaffected by the charge, hence one may speak of the charged membrane as an "ion sieve".

For *high* values of the membrane charge ($\omega\bar{X}/a > 10$) one gets approximately

$$\phi^{\pm} = -\frac{RT}{\delta}\cdot\frac{2uv}{u+v}\cdot(c_2^{+} - c_1^{+}) \qquad \ldots\ (13)$$

$$= -\frac{RT}{\delta}\cdot\frac{2uv}{u+v}\cdot(a_2^{+}\cdot r_2 - a_1^{+}\cdot r_1) \qquad \ldots\ (14)$$

which has a similar form as the NERNST form of FICK's law for a 1-1 valent salt, (15), with the diffusion coefficient $D = 2RT\,(uv)/(u+v)$.

For the opposite case where the membrane charge vanishes, ($\omega\bar{X} \to 0$), one gets $r_2 = r_1 = 1$ and the flux is simply

$$\phi^{\pm} = -\frac{RT}{\delta}\cdot\frac{2uv}{u+v}\cdot(a_2^{+} - a_1^{+}) \qquad \ldots\ (15)$$

2. *Equal total concentrations* in the solutions bathing the membrane, ($a_1 = a_2$), represents a case of special importance for animal physiologists, because this condition prevails in the animal structures. It can be shown that K^{+} in equation (7) transforms to $(RT/\delta)\,(ln\xi)/(\xi - 1)$, hence

$$\phi^{+\prime}_{(a_1 = a_2)} = -\frac{RT}{\delta}\cdot u'\cdot\frac{\ln\xi}{(\xi - 1)}\,(a_2^{+\prime}\xi - a_1^{+\prime})\cdot r \ldots\ (16{:}1)$$

$$\phi^{-\prime}_{(a_1 = a_2)} = -\frac{RT}{\delta}\cdot v'\cdot\frac{\ln\xi}{(\xi - 1)}\cdot(a_2^{-\prime} - a_1^{-\prime}\xi)\cdot 1/r \ldots\ (16{:}2)$$

Here the DONNAN ratio r is a function of $(\omega\bar{X}/a)$ according to equation (8:2) and ξ is a function of the internal (= total) membrane potential as $(\varphi_2 - \varphi_1) = (RT/F)\ln\xi$. In the case of *free diffusion* (no current flow) ξ can be calculated from a modified PLANCK formula[49] on p. 327, or it would be obtained from the externally measured, total membrane potential.

Figure 8 (p. 330) illustrates a typical case where a mixture of (0·09 N HCl + 0·01 N NaBr) is diffusing against (0·01 N HCl + 0·09 N NaBr) through a membrane to which is assigned varying ionization or charge ($\omega\bar{X}$). By studying the ϕ curves of Figure 8(*a*) for the various ion species, one observes that

(*a*) *negatively charged* (*anionic*) *membranes increase the flux of the cations and decrease the flux of the anions.* At *high* charges (strongly anionic membrane ($\omega X \gg a$)) the cation flux would increase proportionally to ωX (compare (18)), the anion flux on the other hand, would tend to very small values.

(*b*) *positively charged* (*cationic*) *membranes decrease the cation fluxes and increase the anion fluxes.* Similarly high $\omega\overline{X}$ values would give approximately proportional anion fluxes and vanishing cation fluxes.

These *qualitative* conclusions regarding the ion transport in charged membranes have for long been assumed in the permeability literature. Our theoretical treatment confirms the assumed rules and gives them a tentative *quantitative* foundation suitable for experimental tests.

A simple and interesting procedure for such a test of equation (16) would be the employment of a radio-isotopic ion in one of the equi-concentrated bulk solutions of a single salt, for instance, as a system like $K^{42}Cl$/membrane/$K^{39}Cl$ (or for anion flux measurements the system KCl^{36}/membrane/KCl^{35}). Here the membrane mobilities u_K and v_{Cl} may be equal, although this condition is not necessary, as identical salts are present on the two sides of the membrane. As the interior potential $(\varphi_2-\varphi_1)$ would be zero, ξ becomes 1 and (16) turns to

$$\phi^{+\prime} = -\frac{RT}{\delta}\, u' \,(a_2^{+\prime} - a_1^{+\prime})\, r \qquad \ldots . (17)$$

A still more extreme special case occurs at a *highly negative* membrane ($\omega\overline{X} \gg a$ in (8:2) yields $r \to \frac{\overline{X}}{a}$), hence (17) gets to the limiting value

$$\phi^{+\prime}{}_{(\omega\overline{X}\gg a)} = -\frac{RT}{\delta}\cdot u' \cdot \frac{\overline{X}}{a}\cdot (a_2^{+\prime} - a_1^{+\prime}) \qquad \ldots . (18)$$

In the particular KCl example, in the *initial* stage, the isotope concentrations are $a_2^{+\prime} = 0$ and $a_1^{+\prime} = a$, hence the cation isotope flux would simply be

$$\phi^{+\prime} \approx +\frac{RT}{\delta}\cdot u' \cdot \overline{X} \qquad \ldots . (19)$$

In a *highly* ionized membrane system, or rather when the ratio membrane charge : bulk concentration is high, one would expect according to (19), that the *'exchange flux' of the mobile ions, of a sign opposite that of the membrane charge, would be approximately constant and independent of the outside salt concentrations, but proportional to the membrane charge.* Similarly, one can show that exchange flux of mobile ions of the same sign would tend to zero. The retarding influence of the total concentration a upon a cation *net* flux as read off from (18) is also worth noticing (anions would be accelerated).

Regarding the predictions from (18) and (19), one may state that the assumed condition is extreme, leading among other things to very steep concentration gradients in the thin transition layers between membrane and bulks. This may invalidate the postulate that the 'boundary process' provides a permanent, instantaneous DONNAN-equilibrium and hence the conclusions drawn from (19) would perhaps be unrealistic. We will take up this point later in another section (p. 359).

3. *Relation to previous work*—It should be emphasized that several of the expressions given above as straightforward corollaries of the new general theory have also been obtained by other authors, although sometimes from somewhat arbitrary assumptions, or highly simplified derivations. GOLDMAN[40] assumed that the membrane contains a large number of dipolar ions near the iso-electric point and that these can act to minimize

the distortion of the electrical field, which would lead to an approach of a situation in which *the field is constant* (*i.e.* $d\varphi/dx$ = constant in our notations). Under such circumstances one may integrate the NERNST-PLANCK equation (1) directly. This tacit assumption was also made by HODGKIN and KATZ[45] and by KEYNES[46], who like GOLDMAN, obtained an equation essentially identical with our equation (16). In neither formulas was, however, the influence of the membrane charge considered (*i.e.* $\omega\bar{X}$ was zero and $r = 1$), although HODGKIN and KATZ in their equations introduced arbitrary "partition coefficients" for each ion species. ERIKSSON[32], on the other hand, introduced the membrane charge. He started from the TEORELL-MEYER-SIEVERS concepts and made a number of simplifying, special assumptions (including the validity of FICK's simple diffusion law, identical mobilities of all mobile ions, absence of interior membrane potential and *high* membrane charge), whereby he arrived at an expression essentially identical with the *special case* (18).

ELECTRICAL PROPERTIES OF IONIC MEMBRANES

As already pointed out, in previous works on 'charged membranes' it has been the electrical membrane potentials, which have attracted almost the entire interest, a fact which may be explained partly by the access to relatively simple methods of experimentation. The original presentations by TEORELL-MEYER-SIEVERS of the 'fixed charge theory' for membrane diffusion were also confined entirely to the development of a quantitative formulation of the over-all membrane potential and its components. The original theory is, however, not satisfactory, because it does not permit a rational solution of the basic process *i.e.* the kinetics of ion fluxes across the membrane (compare p. 317). This fact necessitated a revision and improvement of the original concepts, which have resulted in formulations of ion fluxes (outlined in the previous section). The new theoretical approach has also given new and extended information on the electrical properties of charged or uncharged membranes, not only in cases of free diffusion but also under the application of electrical current, which will now be considered. As before, the presentation rests on a recent paper (TEORELL[19]).

1. *The Membrane Potential*

Components—The essential feature of the original fixed charge theory was the assumption that the over-all membrane potential was composed of three potential jumps : two DONNAN potentials at each interface solution-membrane (here denoted π_1 and π_2) and one residing inside the membrane (the 'internal potential' or 'driving potential' (denoted $\varphi_2 - \varphi_1$)). These

potential jumps are illustrated in Figure 6. The over-all, 'total' membrane potential (here called '*Tot.P.D.*') is the sum

$$Tot.P.D. = (\pi_2 + \pi_1) + (\varphi_2 - \varphi_1) \qquad \ldots . (19{:}1)$$

boundary potentials (DONNAN) — internal potential (HENDERSON PLANCK)

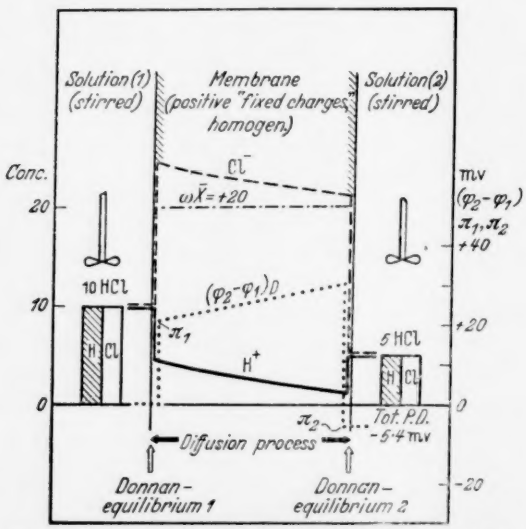

Figure 6. The fixed charge theory applied to a single electrolyte. (A calculated case of HCl diffusing across a positive membrane, the mobility ratio $u_H/v_{Cl} = 5{\cdot}3$). The diagram shows the course of the ion concentrations and of the electric potential. Note here that the Total P.D. has the opposite sign to the interior membrane potential $(\varphi_2 - \varphi_1)_D$. Observe also the non-linear course of the total concentration, (Cl^-), within the membrane. (In this particular case the variation of the potential $(\varphi_2 - \varphi_1)_D$ is approximately linear, which, however, is rather an exception).

The calculation of the sum of the opposing DONNAN *potentials* (in mV at 20°) is easy according to $\pi_1 = -58 \log r_1$ and $\pi_2 = 58 \log r_2$ and yields

$$(\pi_2 - \pi_1) = 58 \log (r_2/r_1) \qquad \ldots . (20{:}1)$$

where the DONNAN distribution ratio r is equal to

$$r = \sqrt{1 + \left(\frac{\omega\bar{X}}{2a}\right)^2} - \left(\frac{\omega\bar{X}}{2a}\right) \qquad \ldots . (8{:}2)$$

ERIKSSON[47] has recently combined (20) and (8:2) into a different form (negative membrane) as

$$(\pi_2 + \pi_1) = 25{\cdot}6 \left[\sinh^{-1} \frac{\bar{X}}{2a_2} - \sinh^{-1} \frac{\bar{X}}{2a_1} \right] \quad \ldots . \text{(20:2)}$$

The version (20:2) may have advantages over the identical (20:1), when discussing relations to the theory of the diffuse double layer.

A compact version of the expression (19:1) for the over-all potential can now be obtained by substituting $(\varphi_2 - \varphi_1)$ by the auxillary potential function $(RT/F) \ln \xi$, whence

$$Tot.P.D. = (RT/F) \ln \left(\frac{r_2}{r_1} \cdot \xi \right) \quad \ldots . \text{(19:2)}$$

The evaluation of the *internal potential* $(\varphi_2 - \varphi_1)$ for a quite *general* case can not be performed correctly by the readily handled HENDERSON formula as in the original derivations for reasons concerning the probable ion distributions within the membrane, that have been mentioned on p. 317. Instead of the HENDERSON picture of 'linear mixtures' within the membrane, the new treatment has employed PLANCK'S concepts of a 'constrained diffusion layer'. The mathematical treatment according to the PLANCK principles is quite elaborate, but one is rewarded by results of more general validity, which certainly come closer to the real physical conditions. The modifying influence of differences in ion activity has also to be considered in further developments of these theories (compare a recent treatment by SCHLÖGL and HELFFERICH[159]).

The extended PLANCK *formula*—The *general* expression for the internal potential, valid for *any* composition of the bulk solutions ($a_1 \neq a_2$), is transcendental and can be written in various forms (compare TEORELL[19] Gl. (7) and Gl.(8)). If we here confine our considerations to the case of *free diffusion* (*i.e.* without electric current flowing), one can show that the *internal membrane potential* can be defined by the following set of equations:

$$(\varphi_2 - \varphi_1)_D = (RT/F) \ln \xi \quad \ldots . \text{(21)}$$

Here ξ has to be determined from the following set of equations

$$\bar{k} = \frac{c_2^+ + 0{\cdot}5\,(1+q) \cdot \omega \bar{X}}{c_1^+ + 0{\cdot}5\,(1+q) \cdot \omega \bar{X}} \left(\text{or } \frac{c_2^- - 0{\cdot}5\,(1-q) \cdot \omega \bar{X}}{c_1^- - 0{\cdot}5\,(1-q) \cdot \omega \bar{X}} \right) \quad \ldots . \text{(22:1,2)}$$

$$\ln \xi = q \cdot \ln \bar{k} \quad \ldots . \text{(23)}$$

$$\frac{\bar{U}_2 \xi - \bar{U}_1}{\bar{V}_2 - \bar{V}_1 \xi} = \frac{c_2^+ \xi - c_1^+}{c_2^- - c_1^- \xi} \cdot \frac{\ln \bar{k} - ln\ \xi}{\ln \bar{k} + ln\ \xi} \quad \ldots . \text{(24)}$$

where

$$\overline{U} = (c^{+\prime} \cdot u' + c^{+\prime\prime} \cdot u'' + \ldots) \text{ and } \overline{V} = (c^{-\prime} \cdot v' + c^{-\prime\prime} \cdot v'' + \ldots) \quad (25)$$

As (24) is transcendental, one has to find ξ by some method of trial and error.

This is done by assigning, in successive steps, arbitrary, different values of the auxiliary term q in (22) ('forbidden' values are, however, those in the range from $-(c_1^+ + c_1^-)/\omega\overline{X}$ to $-(c_2^+ + c_2^-)/\omega\overline{X}$). The corresponding ln ξ and ξ values are now obtainable by aid of (23) and inserted in (24). The procedure is repeated with a new q until left and right member of (24) becomes equal. The corresponding ξ substituted in (21) yields finally the desired $(\varphi_2 - \varphi_1)_D$.

It is of interest to note that equation (24) can be regarded as *an extension of* PLANCK'S *well known formula* for the common diffusion potential. The distinction lies in the fact that the extended form (24) is valid also for homogeneously charged membranes and contains the concentrations of mobile ions of the membrane *interfaces* (the *c:s*). Remembering the assumption that $c^+ = a \cdot r$ and $c^- = a/r$ one realises readily that (24) applied to *uncharged* membranes yields the original PLANCK formula ($\omega\overline{X} = 0$ leads to $r_1 = r_2 = 1$ and hence $\overline{U}$ and $\overline{V}$ become the U and V of PLANCK *etc.*).

In cases where an *external voltage* is applied to the membrane system, the DONNAN potential component $(\pi_2 + \pi_1)$ has to be subtracted from the (measured) over-all membrane voltage in order to obtain $(\varphi_2 - \varphi_1)$ and the corresponding ξ (a knowledge of ξ may be necessary for the handling of, for instance, the flux equations under current flow).

As the DONNAN potential components π can be regarded as equilibrium potentials they are without direct effect on the kinetics of ion penetration. The 'interior potential', on the other hand, enters in all flux expressions as $(\varphi_2 - \varphi_1)$, or as the potential function ξ. Therefore one might venture to call it also a 'driving potential'. A more critical discussion on these matters will be deferred to p. 350.

Special cases—The *internal membrane potential* or the 'driving potential' $(\varphi_2 - \varphi_1)$ can be described by rather simple formulations under some special conditions of free diffusion, which are commonly met with:

(*a*) *A single* 1-1 *valent salt* in different external concentrations (a_1 and a_2). In this particular case it can be proved that the extended PLANCK formula (21)—(24) takes the following form

$$(\varphi_2 - \varphi_1)_D = \frac{u - v}{u + v} \cdot \frac{RT}{F} \cdot \ln \frac{c_1^+ u + c_1^- v}{c_2^+ u + c_2^- v} \quad \ldots\ldots (26{:}1)$$

$$= \frac{u - v}{u + v} \cdot \frac{RT}{F} \cdot \ln \frac{a_1 (r_1 u + v/r_1)}{a_2 (r_2 u + v/r_2)} \quad \ldots\ldots (26{:}2)$$

The formula (26) is, in fact, identical with the HENDERSON component potential proposed, in a different form, in the author's tentative theory of 1935[25,26] and in MEYER and SIEVERS papers of 1936[27,29]. The present result now proves the correctness, for this particular case, of our assumption, which at that time was purely empirical. In Figure 7 is given a

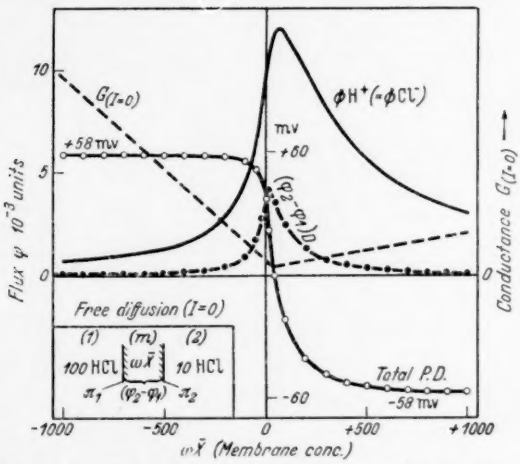

Figure 7. A case of a single electrolyte, HCl, diffusing across an ionic membrane ($u_H/v_{Cl} = 4{\cdot}8$). The diagram shows the net flux of HCl, the interior membrane potential $(\varphi_2 - \varphi_1)_D$, the total potential difference, and the conductance as a function of the charge $(\omega\bar{X})$, of the membrane.

graphical representation of $(\varphi_2 - \varphi_1)$ as a function of varying membrane charge $(\omega\bar{X})$. It is interesting to note that the driving potential $(\varphi_2 - \varphi_1)$ *decreases towards vanishing values when* $\omega\bar{X}$ *becomes high.* This fact and the simultaneously diminishing concentration gradients of the mobile ions inside the membrane explains why the *net* flux of the single salt across a membrane becomes smaller in membranes of increasing charge (compare the statements on p. 324).

(*b*) *Membrane potential at equal external total concentrations* ('*Bi-ionic*' potentials)—When the *total* concentrations a_1 and a_2 are equal, it can be shown that the generalized PLANCK formula (24) transforms into the simple form (valid for *free diffusion*)

$$\xi_D = \frac{\bar{U}_1 + \bar{V}_2}{\bar{U}_2 + \bar{V}_1} \text{ or } \frac{rU_1 + V_2/r}{rU_2 + V_1/r} \qquad \ldots . (27)$$

which again for $\omega\bar{X} = 0$ (uncharged membrane $r_1 = r_2 = 1$) reduces to the classical PLANCK formula $\xi_D = (U_1 + V_2)/(U_2 + V_1)$. In this case the

DONNAN potentials π_2 and π_1 cancel and the *total membrane potential* becomes equal to $(\varphi_2 - \varphi_1)_D$, or

$$Tot.P.D. = \frac{RT}{F} \cdot \ln \left(\frac{rU_1 + V_2/r}{rU_2 + V_1/r} \right) \qquad \ldots . (28)$$

$$U_1 = (a^{+\prime} \cdot u' + a^{+\prime\prime} \cdot u'' + \ldots) \text{ and } V_1 = (a^{-\prime} \cdot v' + a^{-\prime\prime} \cdot v^{-\prime\prime} + \ldots)$$

The influence of the membrane charge is here expressed by r. For the case of highly *positive* membranes $r \to 0$ and the potential will be

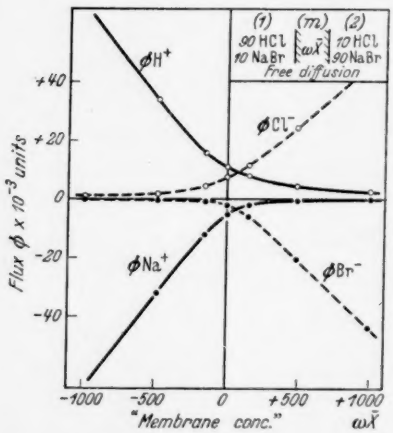

Figure 8*a*. The individual ion fluxes in a system with equal total bulk concentrations, $(a_1 = a_2)$, as a function of the membrane charge. The mobilities are assumed to be those in free water.

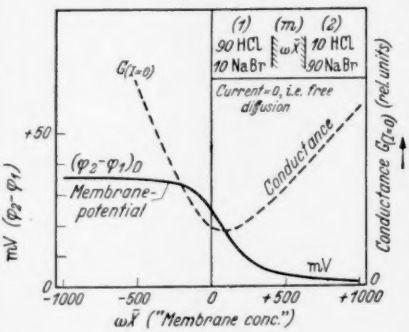

Figure 8*b*. The total membrane potential $(\varphi_2 - \varphi_1)_D$ and total conductance in a system with equal total bulk concentrations $(a_1 = a_2)$ as a function of the membrane charge (the same case as in Figure 8*a*). In this case the DONNAN phase boundary potentials are equal and of opposite signs and cancel, hence the Tot.P.D. is determined solely by $(\varphi_2 - \varphi_1)_D$.

determined by the ratio of V_2/V_1 of the highly permeable anions and correspondingly for *negative* membranes by the cationic ratio U_1/U_2. This means that the conductance is carried exclusively by the anions and cations respectively.

When the system is of the simple type HCl-membrane-NaCl with the anion in common $V_2 = V_1$, and the potential at *positive* membranes will approach zero with increasing membrane charge. Correspondingly at *negative* membranes a limiting value may be attained, determined only by the cation mobility ratio u_H/u_{Na}. As these mobilities may differ much more in the membrane than in 'free water', *the resulting membrane potential may attain any value*, high or low as the case may be (GREGOR and SOLLNER[48], who used the term 'bi-ionic' potentials, found in a chain 0·1 *M* KCl ‖ 'permselective' membrane ‖ 0·1 *M* LiCl a potential of the order +40 to +60 and more millivolts. Further discussion on

mobilities will be deferred to next section). A worked-out case in Figure 8*b* (p. 330) may serve as an illustration to the conclusions drawn.

A formula of the significance of (28) was suggested previously by TEORELL[49]. GOLDMAN[40] (Eq. 18), and HODGKIN and KATZ[45] (Eq. 40), who worked directly with the 'constant field' assumption (compare p. 325) have also obtained expressions similar to (28), although without explicit regard to the membrane charge (our r).

The 'concentration effect', 'apparent ion mobilities' and 'reversible membrane electrodes'—Figure 9*a* gives a diagrammatic example of the over-all

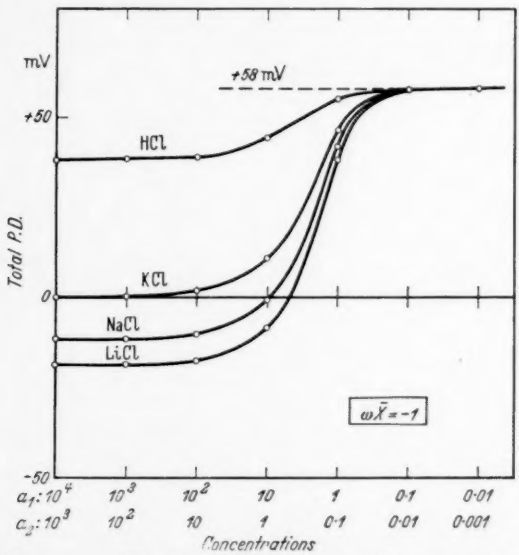

Figure 9*a*. The total membrane potential at a negative membrane with different bulk concentration levels of a single electrolyte of a constant ratio, $a_1 : a_2 = 10:1$, ('the concentration effect'). The mobilities are assumed to be the same as in free water.

potential across an anionic (negative) membrane with different outside concentrations of a single salt, always in a *constant ratio* of 10:1. The curves are calculated according to the formulas in section (a) above with the important assumption that the ion mobilities, u and v, remain the *same* within the membrane as in free water.

The striking feature that the potential varies with the concentration level has been observed repeatedly also on biological objects and has been called 'the concentration effect'. Another variant is pictured in Figure 9*b* and has been seen on frog skin. TEORELL[26] suggested that the frog skin potential behaviour would be due to a charge effect and GREVEN[50] has actually shown this in experiments. LINDERHOLM[64] (p. 78) however,

regards an 'active transport' of Na ions as a possible alternative explanation.

In the past, this effect has often been ascribed to changes of the ion mobilities brought about by the membrane, which should be dependent on the concentrations. The 'relative' or 'apparent' mobilities u/v, or 'transference numbers' have been calculated from the NERNST formula $(\varphi_2-\varphi_1)=(u/v-1)(u/v+1)(RT/F)\ \ln(a_1/a_2)$. The use of the simple

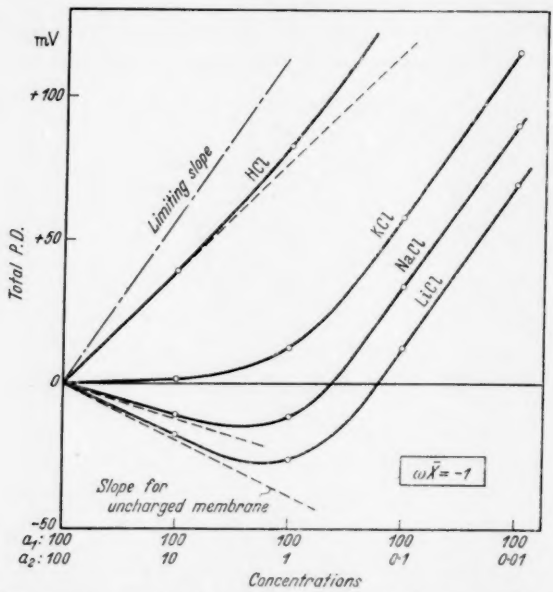

Figure 9*b*. The total membrane potential at a negative membrane of a single electrolyte with a constant bulk concentration (a_1) in (1) and varying concentration (a_2) in (2). The mobilities are assumed to be the same as in free water.

NERNST formula for calculations of 'apparent mobilities' may, however, lead to misleading results and is certainly often unjustified[9,24]. There need not be any marked change of the mobility relations, the 'concentration effect' could be more rationally explained in terms of a change of total, driving force exerted by the fixed membrane ions, as is implied in the equations (26), rather than in terms of mobility changes. As actual examples on current views exposed in this field we may refer to JENNY *et al.*[51], who operate with a concentration dependent transference number and ERIKSSON[47], who refutes this idea and claims that JENNY's results could be as easily explained by the fixed charge theory (as early suggested by the author[24]). Graphical methods for the estimation of the relative mobility u/v and the membrane charge (the 'selectivity constant') have been developed by MEYER *et coll.*[27,31] according to the demands of the fixed charge theory.

As the interior potential $(\varphi_2 - \varphi_1)$ tends to vanishing values *i.e.* $\xi \to 1$, in single salt solutions surrounding a *highly charged* membrane, one would anticipate that the sum of the two DONNAN phase boundary potentials $(\pi_2 - \pi_2)$ would control the over-all potential *i.e.* from (19:2) one gets approximately *Tot. P.D.* $\cong 58 \cdot \log (r_2/r_1)$ millivolts. But we have also *limes* $(r_2/r_1) = a_2/a_1$ when $\omega \bar{X} \to + \infty$ and *limes* $(r_2/r_1) = a_1/a_2$ when $\omega \bar{X} \to - \infty$, whereby

$$Tot.P.D. \cong \mp 58 \log a_1/a_2 \qquad \ldots . (29)$$

This expression is of the same form as the well known formulas for 'reversible electrodes'. For a concentration (activity) ratio of 10:1 of the salt solutions one would accordingly approach the 'thermodynamical maximum' of the membrane potential of about $+58$ or -58 mV as is illustrated in Figure 7 and 9*a*. This is, in fact, what has been observed in experiments on artificial membranes by a great number of authors (MICHAELIS *et al.*, WILBRANDT [for literature see [5]], SOLLNER[6] *etc.*) MARSHALL[52] had already in 1939 tried natural ion exchangers (zeolitic minerals) for measurements of cation activities (compare also WYLLIE[53]; WYLLIE and PATNODE[54]; MANECKE[156]).

Possible biological implications—The potential formation in charged membranes, as discussed above, may perhaps have biological counterparts. We have seen that a variation of the charge of the membrane may result in change of the total over-all potential. In the living systems such a variation may be brought about by *chemical reactions operating upon the membrane matrix thereby inducing an 'action potential'* and the accompanying 'permeability' changes.

2. *Electrical conductivity of membranes*

Introductory remarks—Since the pioneer works of OSTERHOUT in the early twenties, conductivity measurements have often been used in efforts to characterize biological permeability. In recent years the conductivity properties of membrane systems have attracted increased attention, particularly due to the excellent work of a Cambridge group of neurophysiologists (HODGKIN, HUXLEY and KEYNES, compare the review of HODGKIN[55]). The study of the artificial ion exchange materials has also stimulated this discussion. The Cambridge group has in fact already derived a number of theoretical expressions for membrane conductivity, but they have not expressively considered the influence of the intrinsic charges of the membrane matrix. We will therefore, for the sake of consistency, give a comprehensive account of the relevant problem in line with our preceding treatment of the extended fixed charge theory.

The measurement of the conductivity of membrane systems with the conventional *AC* or *DC* methods requires special attention, owing to some particular features not met with in bulk electrolyte solutions. Disregarding the technical difficulties, one has to observe that a diffusion membrane consists of an ion solution of a spatially different composition, which may be subject to change by the measuring current, particularly if *DC* is employed. This property classifies membrane systems as 'non-linear' resistances (compare COLE[78], TEORELL [35,56]). The ideal method would be to use a low alternating current of *high* frequency, provided possible reactance effects arising from double layer capacities *etc.* could be ruled out, or otherwise a very weak *DC*. Under such circumstances one would measure the theoretical resistance Ω (being the inverse value of the conductance G) per unit area as (compare LABES[57]):

$$\Omega = 1/F^2 \cdot \int_{(1)}^{(2)} \frac{dx}{\bar{U} + \bar{V}} \qquad \ldots . (30)$$

An integration of (30) offers considerable difficulty in more general cases of electrolyte mixtures, particularly at charged membranes. Some form of knowledge of the spatial ion distribution is of primary importance here and it is not easy to obtain this (compare p. 355). Another, more convenient and rational principle of defining conductivity of a membrane takes advantage of its 'non-linear' character. For non-linear resistances one cannot apply OHM's law $V/I = \Omega$ *i.e.* there is no constant ratio between voltage V and resulting current I. The time factor, which is also important here, will be discussed later (p. 341). For non-linear systems one instead has to introduce the *dynamic resistance* (compare [58], p. 666) defined as dV/dI, or in terms of total *conductance* (G)

$$G = -\frac{dI}{dV} \qquad \ldots . (31)$$

In (31) the total *current density*, I, is the sum of all *partial* net fluxes, expressed in electrical units as

$$I = F(\overset{i}{\Sigma}\phi^+ - \overset{i}{\Sigma}\phi^-) \qquad \ldots . (32)$$

The various partial net fluxes (ϕ', ϕ'' to ϕ^i) are previously defined by the general net flux equation (9), or by any of its special cases. The voltage V is equivalent to our expression $(\varphi_2 - \varphi_1)$ *i.e.* the internal membrane potential difference, prevailing between interfaces (1) and (2), when a

steady state current is flowing. As before it is useful to transcribe $(\varphi_2 - \varphi_1) = (RT/F) \ln \xi$, whereby (31) becomes

$$G = -\frac{F^2}{RT} \cdot \frac{d\,(\overset{i}{\Sigma}\phi^+ - \overset{i}{\Sigma}\phi^-)}{d\,(\ln \xi)} \qquad \ldots\ldots (33)$$

The influence of varying membrane voltages on the individual net fluxes and the total current density calculated by aid of formulas (16) and (32), is illustrated for a concrete example in Figure 10 (here $a_1 = a_2$).

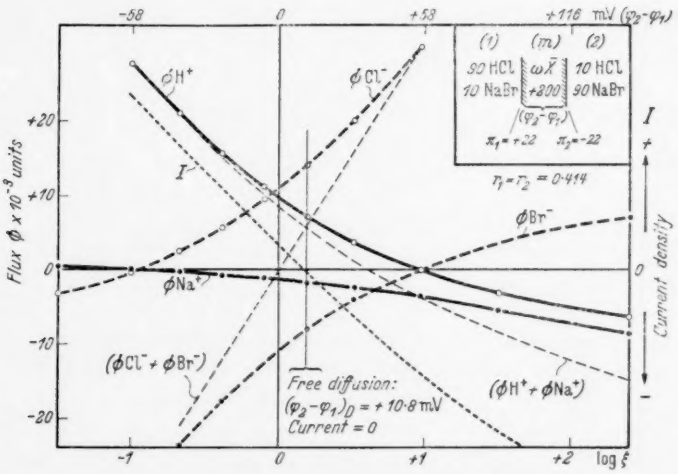

Figure 10. The influence of externally applied potentials on the individual ion net fluxes (ϕ) and on the resulting total current density (I). The system has equal total concentrations $a_1 = a_2$, and is the same as depicted in Figure 8*a* and 8*b*, but the membrane charge is fixed at $\omega X = +200$. Here $(\varphi_2 - \varphi_1)$ represents the resulting potential drop inside the membrane. The slopes of the I-curve are proportionate to the total conductances (the scale is arbitrary). At the potentials + 56 mV and —56 mV there exist 'flux equilibrium' (*i.e.* net flux = 0) for H^+, Br^- and Na^+, Cl^- respectively (compare p. 354).

One observes that I, as expected, varies in a non-linear fashion. The condition $I = 0$ *represents the free diffusion case* and $(\varphi_2 - \varphi_1)$ is accordingly the internal diffusion potential, $(\varphi_2 - \varphi_1)_D$. In free diffusion of course the total cation net flux is equal to the total anion net flux, or $(\overset{i}{\Sigma}\phi^+ = \overset{i}{\Sigma}\phi^-)$.

An explicit expression of G can always be obtained by appropriate differentiation of (33); sometimes graphical methods may be convenient. *Partial* conductance *i.e.* the fraction of G 'carried' by a single ion species, can of course be obtained from (33) by omission of all fluxes but that of the ion of interest, compare LINDERHOLM[64]. We shall here confine our

presentation to a few important *special* cases of *total* conductance at *charged* membranes (compare TEORELL[19] and HODGKIN[55]).

The total conductance in special cases—

(*a*) The biologically important case of *equal total concentrations* in the surrounding bulks (*i.e.* $a_1 = a_2$) conforms to the following expressions

Free diffusion ($I = 0$):

$$G_D = \frac{F^2}{\delta} \cdot \frac{(\bar{U}_2 + \bar{V}_1)(\bar{U}_1 + \bar{V}_2)}{(\bar{U}_1 + \bar{V}_2) - (\bar{U}_2 + \bar{V}_1)} \cdot \ln \xi_D \qquad \ldots . (34$$

With current flow steady state

$$G_{(I \neq 0)} = \frac{F^2}{\delta} \cdot \frac{(\bar{U}_2 + \bar{V}_1) \cdot \xi}{(\xi - 1)} \left[\frac{(1 - \xi_D)}{(1 - \xi)} \cdot \ln \xi + (1 - \xi_D/\xi) \right] \qquad (35)$$

Here again $\bar{U} = rU$ and $\bar{V} = V/r$ ($U = (a^{+\prime} \cdot u' + a^{+\prime\prime} \cdot u'' + \ldots .)$ and $V = (a^{-\prime} \cdot v' + a^{-\prime\prime} \cdot v'' + \ldots .)$), and $\xi_D = (\bar{U}_1 + \bar{V}_2)/(\bar{U}_2 + \bar{V}_1)$ according to (27). It is of interest to note that F^2/δ times $(\bar{U}_1 + \bar{V}_2)$ or $(\bar{U}_2 + \bar{V}_1)$ signifies the 'limiting conductances' (compare p. 340). Incidentally, it can be shown that a membrane 'polarized' or 'short circuited' to a potential of zero *i.e.* $\xi = 1$ in (35), yields a conductance equal to arithmetic mean of the limiting conductances. The equation (34) is equivalent to one derived by HODGKIN and KATZ[45], (Eq. 60); the latter however, lacks the factor r depending on the membrane charge.

The course of the membrane conductance in *free diffusion* is depicted in Figure 8*a* (p. 330) at varying membrane ionization ($\omega \bar{X}$). A study of the figure shows that *the conductance of a charged membrane generally increases with increasing charge* (*ionization*) when all other conditions remain constant.

For the simplest case of a membrane homogeneously inbibated with *a single salt solution*, (35) transforms into $G = (F^2/\delta)(c^+ \cdot u + c^- \cdot v)$ or

$$G_{(I \neq 0)} = \frac{F^2}{\delta} \cdot a (ru + v/r) \qquad \ldots . (36)$$

An analysis of expression (36) reveals that here too G *increases with the membrane charge*. However, a minimum condition with $G = (F^2/\delta) \cdot 2\sqrt{vu}$ exists at $\omega \bar{X} = a(1/r - r)$ where $r = \sqrt{v/u}$ (compare [19], footnote 27). The significance of the special case (36) is immediately obvious and could in fact have been written down off hand: *the conductance is the sum of the contributions of the mobile membrane ions*. Formulas of the type (36) have independently been published recently by SCHMID and SCHWARZ[61] (compare also SCHMID[59,60,62]).

(*b*) When a *single salt* occurs in different bulk concentrations ($a_1 \neq a_2$) the *G*-equation is rather bulky and will be omitted here (compare TEORELL[19], *Gl.* (34)). A worked-out example was, however, depicted in Figure 7 for a postulated case of 0·1 *N* HCl || membrane || 0·01 *N* HCl which again demonstrates that *G* in general should increase with $\omega\bar{X}$.

Experiments on membrane conductance—SCHMID *et al.* have made extensive experiments on specially treated collodion membranes of different 'Festionen-Konzentrationen' impregnated with KCl solution. SCHMID and SCHARWZ emphasize the experimental observations that charged membranes have an unexpectedly high conductivity (which incidentally also has been noted earlier by SOLLNER *et al.*[86,157,158] on 'permselective' collodion membranes). They regard the observations as satisfactorily explained by the fixed charge theory according to TEORELL-MEYER-SIEVERS and discuss the possible influence of other factors as surface conductance, form factors *etc.* MANECKE and BONHOEFFER[63] also used a single salt formula of type (36) on experiments with membranes of anion exchange resins (KCl solutions) and obtained a good agreement with the theory if experimentally determined mobility values were employed. The value for v_{Cl} was about 1/4 of that in free water.

The relations between ionic fluxes and conductance—We are now in a position to be able to describe, if desired, in a quantitative manner the interrelations between ionic 'permeability' and the results of electrical conductivity measurements. Both the individual ionic net fluxes and the total (or partial) conductances have been defined above in terms of constant ion mobilities, ion concentrations and the membrane charge. Apparently one can combine the formulas in a variety of ways in order to conform to the conditions of particular interest. A number of useful relations, applicable to uncharged membranes, can be found in HODGKINS[55] paper, compare also LINDERHOLM[64]. As to the conclusions that can be inferred from such procedures the following might be summarized : When dealing with 'neutral' (uncharged) membranes, one generally finds that an increase of any partial ionic flux is reflected in a concomitant rise of the total conductance. Such a parallelism should, however, not be taken for granted in the presence of an ionic membrane. Here it may happen that the net flux of an ion species decreases, while the total conductance increases. The conditions of Figures 8*a* and 8*b* (p. 330) may serve as an example of the last mentioned event. When the positive membrane becomes more positive, the cation fluxes decline, although the total conductance would show a rise. Corresponding observations can be made also in the single electrolyte case of Figure 7 (p. 329). In both examples the *membrane charge is the operative factor*, not necessarily a change of any ion mobility. This behaviour may well occur in biological systems.

3. *Membrane rectification*

Introductory remarks—In Section 2 it has been pointed out that a solution-membrane-solution system behaves non-linearly when subject to an externally applied current *i.e.* the total conductance varies with the current density. Moreover, the conductance will differ according to the direction chosen for the current ('rectification'). When speaking of conductance here we refer to the steady state condition, because the time factor is also of decisive importance. The mechanism of the conductance changes is obviously a spatial redistribution of the ion concentrations within the membrane. When changing from one current or voltage value to another, it will take a finite time to attain a new steady state. The membrane-electrolyte system therefore behaves similarly to what in electrical technology has been called 'time-varying, non-linear resistances' and it would be appropriate to speak of a 'time-varying rectification'[35,56]. The term 'delayed rectification' has also been used (HODGKIN *et al.*[65]).

The rectification phenomenon has been observed for long on various inanimate and animate systems. EBBECKE[66–68] had already observed rectification in nerves, LABES and ZAIN[69–72] found it on a collodion membrane surrounded by different salts, so did BLINKS[73]. More recent papers dealing with rectification are those of GUTTMAN[74–76], COLE and CURTIS[77], GOLDMAN[40], TEORELL[35,56] and HODGKIN *et al.*[65]. In some cases as high as a 20-fold difference in resistance could be observed between the two current directions. GOLDMAN seems to be the first to theoretically approach the phenomenon of membrane rectification. Although he started his general considerations with attention to a possible charge of the membrane, he dropped the charge concept when he dealt with rectification. He tacitly assumes instead a 'constant field' theory, which later on was taken up by HODGKIN and KATZ[45]. As the 'constant field' hypothesis is not off-hand applicable to charged membranes, we have undertaken a new analysis of the rectification problem consistent with the extended fixed charge concepts already applied above to ion fluxes, membrane potentials and conductance. The results have shown that the two methods of approach yield, for the *special* case of equal total concentrations ($a_1 = a_2$), identical results, provided the formulas are expressed in terms of ion concentrations *in* the membrane. This result may serve as a hitherto lacking, indirect proof for the theoretical validity of the constant field assumption when $a_1 = a_2$. As regards *uncharged* membranes, it has previously been pointed out that a straight-forward use of the NERNST-PLANCK concepts in the situation $a_1 = a_2$ leads to an electrical field gradient, $d\varphi/dx$ = constant (TEORELL[35], p. 211). Nevertheless, the GOLDMAN-HODGKIN-KATZ formulas are inapplicable to charged membranes unless they become supplemented with an explicit formulation of

the relations between bulk and membrane interface concentrations (in the following we adopt the DONNAN distribution concept).

Limiting conductances, the 'rectification ratio'—When a sufficiently high steady voltage is applied in one direction to an electrolyte-membrane, a

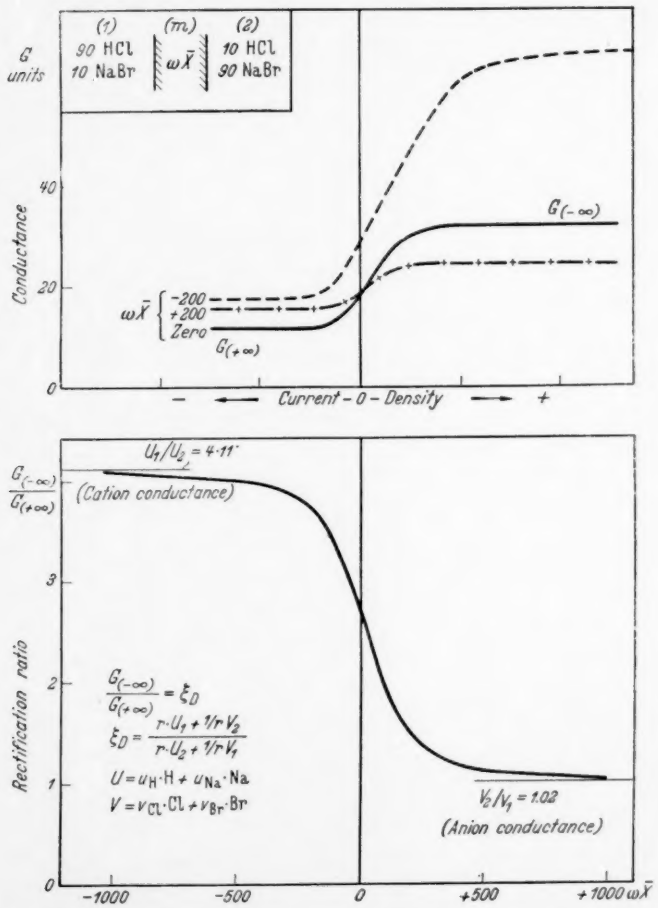

Figure 11. (Top) The influence of the current density on the total conductance (in the steady state). The system has equal total concentrations ($a_1 = a_2$) and is the same as in Figures 8 and 10. Note the 'rectification' present in all three cases of different membrane charge.

(Below) The effect of different membrane charge on the magnitude of the rectification property. $G(_{-\infty})$ and $G\,(_{+\infty})$ denote the limiting conductance at very high negative and positive potential respectively.

state will ultimately be reached when the cations of one bulk solution and the anions of the other will entirely fill up the membrane matrix.

The other ions will be pushed out and a *limiting conductance* value, $G(+\infty)$, will be approached. With reversal of the direction the value will become $G(-\infty)$. We now restrict the discussion to the case $a_1 = a_2$, which corresponds to a common biological condition. The limiting conductances here can be readily obtained by inserting $\xi = +\infty$ or 0 in (35) yielding

$$G(+\infty) = \frac{F^2}{\delta}(\overline{U}_2 + \overline{V}_1) \text{ and } G(-\infty) = \frac{F^2}{\delta}(\overline{U}_1 + \overline{V}_2) \quad \ldots . (37)$$

The ratio $G(-\infty)/G(+\infty)$ is a kind of *measure of the rectification property* of the system, which we obtain from (37) and define as

$$\textit{Rectification ratio} = \frac{\overline{U}_1 + \overline{V}_2}{\overline{U}_2 + \overline{V}_1} = \xi_D \quad \ldots . (38)$$

The 'constant field' theory of GOLDMAN gives for $\overline{X} = 0$ the same result (*loc. cit.*, Eq. (18)). It is interesting to note that ξ_D is the same auxiliary term that also determines the internal membrane *diffusion* potential as $(\varphi_2 - \varphi_1)_D = (RT/F)\ln\xi_D$ (compare (21)); or expressed more to the point, at equal total concentrations surrounding the membrane, *the rectification runs parallel to the diffusion potential* according to ξ_D or $\exp[F(\varphi_2 - \varphi_1)_D/RT]$. The $\xi_D = 1$ means no rectification, $\xi_D \gtrless 1$ indicates rectification. This statement also gives an answer to the question what the membrane charge means to the rectification. The matter may be visualized by inspection of the curves in Figure 11. One observes that *rectification must be a property common to all membranes, charged or uncharged, due to differences in ionic mobilities, although a charge may increase or decrease this property.*

At high relative membrane charge values the terms $\overline{U} = rU$ or $\overline{V} = V/r$ vanish for positive, respectively negative membranes, (because r tends to zero, or to $+\infty$). This signifies that *the electric conductance in highly charged membranes would be maintained solely by the oppositely charged, mobile 'gegen' ions*, a rather obvious conclusion.

4. *Reactance properties of membranes*

Introductory remarks—Numerous investigations, particularly on living membranes, with alternating currents have shown that they exhibit a composite resistance *i.e.* an *impedance*, which is different at different frequencies of the current employed. The leading workers in this field have been LULLIES, GILDEMEISTER and particularly COLE and collaborators. Plain electrolyte solutions, on the other hand, show a frequency independant resistance. The impedance of the membranes has been resolved into a 'resistance' term (of the ohmic type) and a 'reactance' term, the latter being usually regarded as due to a 'capacity' residing in the membrane

structure. Many speculations have been devoted to the significance of these impedance terms (for comprehensive papers, see for instance DAVSON and DANIELLI[2], Chapter XV.) Although it is true that the

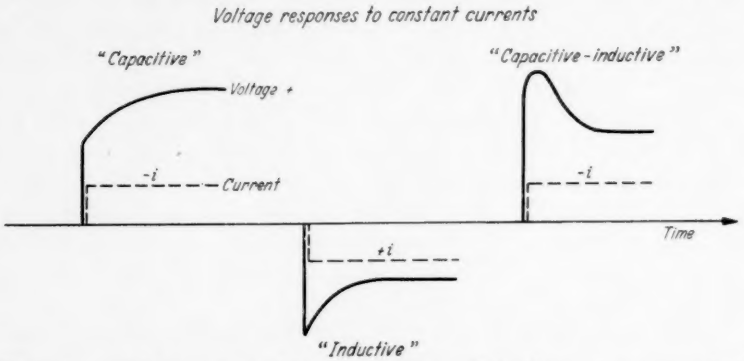

Figure 12. Scheme of possible time-voltage relationships of asymmetrical electrolytes-membrane systems (after application of a constant current). Note the different voltage responses indicating *apparent* capacitance, inductance or capacitance + inductance.

impedance methods have offered excellent service in permeability research as sensitive indicators that 'something has happened', it is regrettable that no convincing or complete view of its theoretical background has as yet been put forward. A common interpretation has been that the *resistance term* is connected with the *ion permeable* structures and the *capacitance* term with the *ion impermeable* parts (lipoids layers *etc.*) of the membranes or tissues investigated.

This view, however, may at best be only partly correct in the light of some recent papers on 'anomalous' or 'apparent' reactances (COLE [78,79] and TEORELL[19,35,56]). In order to elucidate the nature of these concepts, we will pursue somewhat further the problem of membrane rectification treated in the previous section.

The connections between rectification and reactance (capacitance and inductance)—It has been pointed out above that the asymmetrical system electrolytes-membrane, regardless of whether the latter is carrying a charge or not, must behave as a 'time-varying, nonlinear resistance' and therefore exhibits a 'time-varying' or 'delayed' rectification. When a steady electric current is applied to such a system, it would require some time or delay until the voltage attains a steady value, because the rearrangement of the ionic distribution pattern inside the membrane to the new condition is not instantaneous. These features are illustrated in Figure 12.

The events depicted in this figure are, however, exactly those which would be observed if a real capacity (condensor) or inductivity (inductance coil) had been used instead (complemented with one series and

one parallel resistance). This may suffice to justify the statement, that *membranes with asymmetrical ion distributions are bound to exhibit an 'apparent capacitance'* (when the membrane conductance decreases under the current flow) or '*apparent inductance*' (when the conductance increases).

COLE[78] was the first to anticipate these effects. In some formal considerations of the analogy between the properties of barrier-layer rectifiers and ion-membrane rectification (for a postulated case of exclusive potassium permeability), COLE arrived at the conclusion that 'anomalous reactance has to be expected in non-linear systems' (*loc. cit.* p. 56). Without knowledge of COLE'S paper, the present author suggested a similar concept (TEORELL[56,35]) and also demonstrated experimentally that a sintered glass membrane surrounded by 0·1 *N* LiCl and 0·1 *N* HCl gave an 'impedance locus curve' characteristic for a system containing a very large capacitance and inductance.

A closer analysis of this type of apparent reactances in relation to those observed on other inanimate or animate systems is, however, desirable before one can say something more definite on these matters. A tempting aspect would be to find out the possibilities of an oscillatory behaviour of this system (*cf.* HODGKIN[55], p. 386).

When attempting to discuss the biological reactance phenomena one has to remember that the 'asymmetry-type' of the apparent reactance treated above is only one possible component of these phenomena. The real static capacities of the ion impermeable structures and double layer capacities have also to be considered. The apparent reactances are probably exhibited in the low frequency range, and the double layer and dielectric capacities in the high frequency range, when analyzed in terms of alternating currents.

5. *Flux relations and ionic distribution phenomena*

The biologists have hitherto perhaps been more interested in the ionic 'accumulation' or 'depletion' phenomena so often exhibited in living systems than in the ionic transport kinetics. An example of such an ionic distribution phenomenon is the selective potassium accumulation in the human erythrocytes, where the inside K-concentration is about 16 times higher than that outside in the plasma (with a reversed condition for sodium). Nowadays one is aware that such differences of concentrations are effects of dynamical equilibria, or steady states, and not due to some impermeability of the cell membrane with respect to K or Na ions. In fact, in an increasing number of biological cases it has been revealed that concentration differences of ions across cell and tissue boundaries are the result rather of the play of 'driving forces' than of a specific ability of penetration (*cf.* discussion of false impermeability, p. 308). Pure physico-chemical mechanisms alone are certainly insufficient to explain

many selective ion accumulations and a number of 'active transport' hypotheses have been proposed, involving the expense of chemical energy. Nevertheless, it is of great value for the biologist to investigate inanimate diffusion systems as models for the living matter. In fact, a great number of, at first sight, rather baffling transport phenomena can be observed, which may have biological counterparts. We shall therefore now examine the ionic distribution phenomena that can be anticipated in membrane diffusion systems in line with our previous treatment.

Membrane potentials and ionic distribution equilibria—The classical model system has been the DONNAN equilibrium. Here the complete non-penetrability of one or several ions has been the *conditio sine qua non* for forcing other ions into 'migration against concentration gradients' resulting in an *equilibrium* state characterized by unequal ionic concentrations. For some time it has been realised that similar distribution effects can be expected and demonstrated also in systems where *all* ions are permeable through a membrane, although to a different extent. TEORELL[80] showed theoretically that a steady diffusion of one electrolyte (the active or 'the diffusing agent', D^+A^-) across a membrane ought to result in steady state, with an *equilibrium* distribution of the other ions present (M^+B^-) characterised by the same ratio of concentrations $(M^+)_i/(M^+)_0 = (B^-)_0/(B^-)_i = r$ as is familiar from the DONNAN systems. For those ions which were capable of attaining such an equilibrium the term 'passive ions' was proposed. The DONNAN effect can in fact be regarded as a special case of this more general '*diffusion effect*'. An experimental set up gave a satisfactory verification of the theory (TEORELL[81]). In Figure 13 we reproduce a scheme of a model of the 'diffusion effect' on an uncharged membrane. The mechanism here centres on the simple *diffusion potential* primarily set up by the differences in mobility of the H and Cl ions of the diffusing agent, HCl. One notices how the passive K and Br ions inside the membrane are operated upon simultaneously by the 'electric force' (black arrows) and 'osmotic force' (white arrows) until these forces eventually become equal and a final steady state reached, when the K and Br concentrations inside and outside rest in a pseudo-equilibrium so that $(K^+)_i/(K^+)_0 = (Br^-)_0/(Br^-)_i = \xi$. One may describe this situation by the provisional phrase 'the unequal ionic distributions are maintained by the membrane potential present', because it can be shown that the membrane potential $(\varphi_2 - \varphi_1) = (RT/F)\ln\xi$.

This conception of *individual ionic equilibria superposed on a dynamic system* has gradually penetrated into recent work on ionic processes in biology. At the time of its proposal in 1935 it was by no means self-evident, although it was easy to point out the analogies to a BOLTZMANN distribution *etc.* once the existence of these effects had been demonstrated. Below we will make an attempt to show that some further progress can be made

in extending the original theory of the diffusion effect to include also the influence of *charged* membranes. This attempt must, however, be preceded by some definitions concerning ion fluxes and distribution equilibria.

The net flux, the influx and the outflux—In the steady state of the diffusion effect system just mentioned, or the DONNAN system, as well as *in any*

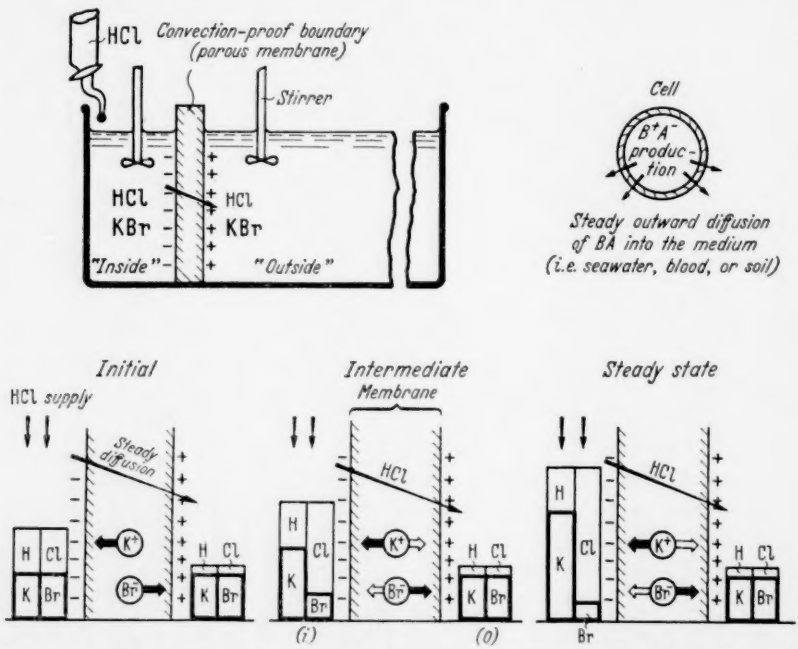

Figure 13. Scheme illustrating the development of a 'diffusion effect' upon the ionic distribution (uncharged membrane). (Top, left) : The experimental set up : the HCl (the 'diffusing agent') is steadily supplied into 'inside' (small volume) and diffuses across the membrane to 'outside' (large volume). An electrical potential across the membrane results. (Top, right) : A hypothetical biological counterpart to the experiment. (Below) : The different stages in the development of the 'diffusion effect' upon the distribution of the 'passive' ions (K and Br). Black arrows indicate electrical force, white arrows indicate 'osmotic' force. In the final steady state these forces balance each other and the K and Br ions attain a 'flux equilibrium'.

system in distribution equilibrium, there is no *net* flux of the passive ions. This is, of course, not due to an immobilisation of the ions by the membrane, but is a consequence of the fact that there exists a never-ceasing inward transport (influx) and a backward transport (outflux) of ions, which just balance each other. Hence, in general one has *net flux* = *influx* − *outflux* and in the steady state a *net flux* = 0, or *influx* = *outflux*.

A quantitative definition of these component fluxes and the resulting ionic distributions for *charged membranes* can be made in consistency with the previous treatment in this paper (based on TEORELL[19]). According to

equation (7) one has for a cation species *net flux* $= -(K^+ \cdot u' \cdot {c_2}^{+'}\xi) + (K^+ \cdot u' {c_1}^{+'})$. It is obvious that the first term represents the 'influx' (in the direction (2) → (1)) and the second term the 'outflux' (direction (1) → (2)) or

$$\text{cation influx} = -K^+ \cdot u' \cdot {c_2}^{+'}\xi \qquad \ldots . (39)$$

$$\text{cation outflux} = +K^+ \cdot u' \cdot {c_1}^{+'} \qquad \ldots . (40)$$

If desired, one can calculate the *absolute* influx and outflux values after a numerical evaluation of K^+ (or K^- for anions) which is always possible, by means of the various equations given in the sections on pages 319 to 324.

A more useful and interesting quantity is, however, a *relative* flux expression obtained by dividing (39) by (40). This may briefly be denoted "in-out flux ratio", written as

$$\textit{cation}\ \frac{(\textit{influx})}{(\textit{outflux})} = -\frac{{c_2}^{+'}\xi}{{c_1}^{+'}} = -\left(\frac{{a_2}^{+'}}{{a_1}^{+'}}\right)\cdot\left(\frac{r_2}{r_1}\cdot\xi\right) \qquad \ldots . (41)$$

$$\textit{anion}\ \frac{(\textit{influx})}{(\textit{outflux})} = -\frac{{c_2}^{-'}/\xi}{{c_1}^{-'}} = -\left(\frac{{a_2}^{-'}}{{a_1}^{-'}}\right)\cdot\left(\frac{r_1}{r_2\xi}\right) \qquad \ldots . (42)$$

Here it is appropriate to recall that according to (19:2) one has a relation to the total membrane potential as *Tot. P.D.* $= (RT/F) \ln (r_2\xi/r_1)$. One could therefore transcribe the in-outflux ratios (41) and (42) in terms of the *Tot. P.D.* as

$$\textit{cation in-outflux ratio} = -({a_2}^{+'}/{a_1}^{+'}) \cdot \exp\,[(\textit{Tot.P.D.})\,F/RT] \quad . (43)$$

and

$$\textit{anion in-outflux ratio} = -({a_2}^{-'}/{a_1}^{-'}) \cdot \exp\,[-(\textit{Tot.P.D.})F/RT] \quad . (44)$$

These equations teach that *the in-outflux ratio of an ion species is determined solely by its bulk concentrations and the over-all membrane potential.*

TEORELL[35,82] and USSING[38] have independently pointed out the existence of this type of flux relation, both, however, on grounds that neglect the charge of the membrane. When this can be neglected $r_2/r_1 \longrightarrow 1$ and one gets from (41) and (42) the formulas employed by TEORELL or USSING in the form

$$\frac{(\textit{influx})}{(\textit{outflux})} = -\frac{{a_2}^{+'}}{{a_1}^{+'}}\cdot\xi \text{ or } -\frac{{a_2}^{-'}}{{a_1}^{-'}}\cdot\frac{1}{\xi} \qquad \ldots . (45)$$

where ξ is the 'potential function' equal to $\exp\,[(\varphi_2 - \varphi_1)\cdot F/RT]$. The 'driving' potential $(\varphi_2 - \varphi_1)$ can either arise from an externally applied electric current, or can simply be identified as the diffusion potential of the system. It should be pointed out that (45) is a direct corollary to a

relation given already in 1897 by BEHN[41] (compare TEORELL[19,35] and the next paragraph).

USSING[37,38,39,83,148] in a series of valuable papers has attempted a distinction between 'active' and 'passive' ion transport in biological systems and has thereby determined in-outflux ratios by aid of radioactive tracers. Similar experiments on nerves are summarized by HODGKIN[55]; compare also LINDERHOLMS[64] work on ion transport in frog skin. USSING as well as TEORELL claims without proofs that (45) is valid 'regardless of the structure of the membrane', or 'regardless of the origin of the potential'. Our derivation above of expressions (43)-(44) offers a formal proof that one really is allowed to calculate the in-outflux ratios for ions behaving passively, also from the measured 'mixed' potential of a *charged* membrane system (at least under conditions conforming to the fixed charge theory as outlined here).

Other flux relations

In experimental work on ion permeability it may be useful to calculate also other flux relations than those mentioned in the previous paragraphs.

(*a*) *Net flux ratios*—The simplest flux relation between various ion species in a membrane transport system is the *net flux ratio* between pairs of ions of the same sign. This can be directly obtained by dividing the two corresponding net flux equations of type (9), whence the common coefficient K^+ for cations (and K^- for anions) cancels out

$$\textit{cationic net flux ratio}\ \frac{\phi^{+\prime}}{\phi^{+\prime\prime}} = \frac{u' \cdot (a_2{}^{+\prime} \cdot r_2\xi - a_1{}^{+\prime} r_1)}{u'' \cdot (a_2{}^{+\prime\prime} \cdot r_2\xi - a_1{}^{+\prime\prime} r_1)} \quad \ldots . \quad (46)$$

$$\textit{anionic net flux ratio}\ \frac{\phi^{-\prime}}{\phi^{-\prime\prime}} = \frac{v' \cdot (a_2{}^{-\prime}/r_2\xi - a_1{}^{-\prime}/r_1)}{v'' \cdot (a_2{}^{-\prime\prime}/r_2\xi - a_1{}^{-\prime\prime}/r_1)} \quad \ldots . \quad (47)$$

As early as 1897 did BEHN[41] derive a similar net flux ratio although in a different manner and under assumptions valid for an *uncharged* membrane (in such a case $r_1 = r_2 = 1$ and (46) becomes identical with the original BEHN formula).

One can of course also compare a net flux for any cation with that of any anion, which, however, requires an explicit evaluation of the ratio between K^+ and K^-. This is a cumbersome procedure for the general case, but gives simple results for the special cases of *equal total concentrations* ($a_1 = a_2$), when $K^+/K^- = 1/\xi$, and the *single salt* case (see below).

(*b*) *Diverse partial flux ratios*—In recent experimental work with radioactively marked ions it has been possible to disclose the dynamic character of various steady state distributions. A small amount of marked ions is added to either side of a membrane and the migration rate over to the other side then determined. Hitherto usually the in-outflux ratios of one and

the same ionic species has been compared *cf. e.g.* USSING or LINDERHOLM. It is, however, possible to gain a more complete idea of the transport conditions in the membranes by a 'cross comparison' between other measurable rates as the ratio (influx rate of a cation) : (influx rate of an

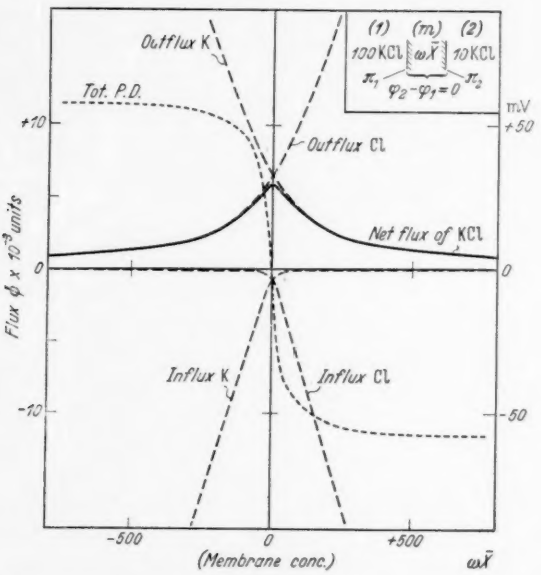

Figure 14. The influence of the membrane charge upon the net flux, outflux and influx of the ions of a single electrolyte. The ionic mobilities are assumed to be identical (KCl). Note the small net flux at high $\omega\bar{X}$ values, (where, however, the influx and outflux may be very large or vanishing,) thus a negative membrane becomes virtually anion impermeable and a positive membrane becomes cation impermeable.

anion) and many other combinations. In Figure 14 is demonstrated how the absolute values of partial fluxes and the net flux may be influenced by the membrane charge (compare also the salt mixture depicted in Figure 8). We have chosen a *single salt* in the concentrations 10:1 separated by a negatively charged membrane. For simplicity it was assumed that all ion mobilities in the membrane were equal. It may now be instructive to make a list of the various ratios between the influxes and outfluxes expressed by equations of the type (41) and (42) for cations and anions respectively In a single salt case one can show that $K^+/K^- = [v' \cdot (c_2^{-\prime}/\xi - c_1^{-\prime})]/[u' \cdot (c_2^{+\prime}\xi - c_1^{+\prime})]$. The expected results for all the possible six partial flux relations are assembled in *Table III*. For comparison the flux ratios expected for an uncharged membrane also are given

Table III. Partial flux ratios at a charged membrane (approximately equal mobilities for all ions present)

	(1)		(2)	
Initial state:	10 mM KBr (500 ml)	Cellophane membrane $\omega\bar{X} \approx -20$ ($\delta = 0{\cdot}3$ mm)	1 mM H_4NCl (12 ml)	
Initial flux: (analytical results in mM/h	*Cations*: Outflux (K)	1·22	*Anions*: Outflux (Br)	0·53
	Influx (H_4N)	0·75	Influx (Cl)	0·02
	Net flux	0·47	Net flux	0·51

Initial potential: + 43·5 mV (*Tot. P.D.*) $\quad \therefore p = e^{\frac{+43{\cdot}5}{58}} = 5{\cdot}6$

Ratio	$\frac{In^+}{Out^+}\left(\frac{H_4N}{K}\right)$	$\frac{In^-}{Out^-}\left(\frac{Cl}{Br}\right)$	$\frac{In^+}{In^-}\left(\frac{H_4N}{Cl}\right)$	$\frac{Out^+}{Out^-}\left(\frac{K}{Br}\right)$	$\frac{In^+}{Out^-}\left(\frac{H_4N}{Br}\right)$	$\frac{In^-}{Out^+}\left(\frac{Cl}{K}\right)$
Formula	$-\frac{a_2 p}{a_1}$	$-\frac{a_2}{a_1 p}$	$\frac{a_2 - a_1 p}{a_2 - a_1/p}$	$\frac{a_2/p - a_1}{a_2 p - a_1}$	$\frac{a_2/a_1 - p}{a_1/a_2 - p}$	$\frac{a_2 p/a_1 - 1}{a_1 p/a_2 - 1}$
Calculated ratio	− 0·57	− 0·017	− 72	+ 2·28	− 1·28	− 0·008
Experimental ratio	− 0·61	−0·04à0·08	−19à—37	+ 2·21	− 1·47	−0·01à0·03
Calculated ratio $\omega\bar{X} = o$	− 0·10	− 0·10	− 1·0	+ 1·0	−0 ·10	− 0·10

(when $\omega\bar{X} = 0$ one obtains $p = 1$). It is obvious from *Table III*, that the negative charge of the membrane greatly upsets the partial fluxes: for example the relations 'resorption' of the cation (H_4N^+) from the dilute solution is enhanced in comparison with the anion (Cl^-) by a factor of 72, indeed a pronounced 'selectivity'. Here H_4N and Br are used as 'isotopes' of K and Cl respectively (almost identical mobilities!).

The row 'Experimental ratio' refers to an actual experiment with a set up given in the table, where also the actually measured initial fluxes are given (unpublished material). The agreement between calculated and observed data can be regarded as satisfactory and lends some support to the correctness of the presented concepts.

Ionic distribution equilibria

If a state is attained when (influx) = (outflux) for an ion species, one may say that it has entered in a dynamic *equilibrium*. By putting the in-outflux ratio of any of expressions (43) to (45) equal to 1 (*i.e.* the *net* flux being zero), one finds that an equilibrium state is possible for any *passive* ion characterized by the same *distribution ratio*, p, as

$$p = \left(\frac{a_1^{+\prime}}{a_2^{+\prime}}\right) = \left(\frac{a_2^{-\prime}}{a_1^{-\prime}}\right) = \frac{r_2}{r_1} \cdot \xi = \exp\left[(Tot.P.D.)F/RT\right] \ldots . \quad (48)$$

These equations imply first the important fact that *a migration against concentration gradients is possible* (for literature see TEORELL[80,81] or MCBAIN[42,43]) and secondly that *any passive ions always will strive to establish an equilibrium state, which is solely defined by the common over-all membrane potential* (*Tot. P.D.*). A condition of accumulation of certain ions and depletion of others will eventually be obtained similar to the one pictured in Figure 13. One necessary provision, however, is that the potential by some means attains a steady level, or, at least, does not decline to zero too fast. The potential may be maintained by a flowing current.

An interesting possibility is that a constant and permanent diffusion of an 'active' or 'diffusing electrolyte' can provide for the maintenance of a constant potential (p. 343). When all *passive* cations and anions have reached equilibrium levels, it will be solely the diffusing agent which flows across the membrane, the net flux of its cations being equal to that of its anions.

In the case, where the *Tot. P.D.* is created by pure diffusion, we have present an extended case of what above has been called the 'diffusion effect' (the original theory[80] was developed somewhat differently and restricted to uncharged membranes). Now, when the ions of the diffusing agent are the only ones flowing, it can be readily shown that the ξ component in the distribution ratio $(r_2\xi/r_1)$ is determined by the 'single salt' formula (26), where the mobilities u' and v' are those of the diffusing ions, a_1 and a_2, on the other hand, refer now to the *total* concentrations in the final steady state. Hence, for the *passive* ions one gets

$$\log\,(\textit{equilibrium distribution ratio}) = \log\,[(r_2/r_1)\cdot\xi] =$$

$$= \log\,(r_2/r_1) + \frac{u_D - v_A}{u_D + v_A}\log\frac{a_1\,(u_D r_1 + u_A/r_1)}{a_2\,(u_D r_2 + v_A/r_2)} \qquad \ldots\ (49)$$

The (r_2/r_1) component is due to the membrane charge (note that $(RT/F)\ \ln\,(r_2/r_1)$ is the sum of the DONNAN potentials). For $\omega\bar{X} = 0$ one gets $(r_2/r_1) = 1$ and obtains from (49) the same formula as derived originally by TEORELL[80,81]. One may also observe that the ratio $(r_2\xi/r_1)$ has a significance similar to the 'partition coefficient' at oil-water systems.

The present, extended treatment can be considered as a kind of rigorous proof of some statements previously made on more intuitive grounds. Thus the author in 1937 (TEORELL[84], p. 941) suggested the generalisation—'The equilibrium towards which a species of charged particles tends may be expressed by

$$\text{electrical work} = \text{'osmotic' work}$$

$$z\varphi F \qquad\qquad RT\cdot\ln\frac{c_1}{c_0}$$

$$i.e.\ c_1/c_0 = \exp\left(\frac{z\varphi}{RT/F}\right) \qquad \ldots\ (50)$$

regardless of the origin of the potential and the nature of the particles and the membrane . . . '. The equilibria concentrations were denoted c, the potential φ, the particle charge (valency) z. It was actually possible to demonstrate this type of distribution equilibria (similar to that of Figure 13) in experiments both with ions (compare TEORELL[81]) and with charged colloid particles (gum mastic) (TEORELL[84], p. 940).

In these early considerations it was said that the membrane potential 'performed work' owing to the difference in concentrations and *mobilities* of the ions of the diffusing agent. However, in the light of the extended considerations implicit in (48) and (49), it would be possible to achieve partial ionic equilibria even *in the absence of any mobility differences and interior potential*, provided the membrane is *charged* and the total concentration is maintained *higher* on one side of the membrane. In such a case would the factor ξ in the distribution ratio $(r_2/r_1)\xi$ be equal to 1 but the factor r_2/r_1 kept $\gtrless 1$ and hence make possible an unequal passive ionic distribution.

In some experiments with a system (1) 0·1 *N* $H_4N\cdot Cl$ (small volume) ‖ charged membrane ($\omega\overline{X} = -20$) ‖ 0·1 *N* $H_4N\cdot Cl$ (2) (large volume) a steady supply of a strong KCl solution as a diffusion agent was added to volume (1) (compare the arrangement of Figure 13). In separate experiments it was found that the membrane mobilities of H_4N^+, K^+ and Cl^- were nearly equal. Nevertheless after some time of KCl-addition a steady over-all potential difference (*Tot. P.D.*) of about +17 mV was measured and the analyses revealed an about 2-*fold* accumulation of the ammonium ions in (1) (unpublished material). This is in excellent agreement with equation (48) where RT/F is equal to 25·2.

The important new view points suggested by the equation (48) and (49), and by the simple experiment mentioned, are the following:

According to our convention as regards the component potentials at the charged membrane, it would have been said that the sum of the two (DONNAN) phase boundary potentials 'performs work' leading to the ammonium accumulation inside the model 'cell'. Such a statement introduces, however, a formal dilemma in so far as it is awkward to speak of two *equilibrium* systems as sources of energy. It seems therefore advisable to drop entirely the suggestive, but misleading phrase, that electrical potentials supply the energy of ionic accumulation. Instead the attention should be focussed on the fact, that the total free energy difference, owing to the total concentration difference, is imparted upon the passive ions, forcing them to redistributions. This concept could certainly be better expressed by other methods than the classical-kinetical one here employed.

'*Temporary distribution equilibria*'

In any asymmetrical electrolyte-membrane system where an over-all potential can arise, one has to expect an ion exchange *tending* towards

an equilibrium according to formula (48). Indeed, it is not necessary to have a continuous supply of a diffusing agent to achieve 'accumulation' effects. In *Table IV* we illustrate such an event, taken from a model experiment (unpublished).

Table IV. Development of a temporary ionic distribution equilibrium in a diffusion system with a charged membrane (all ions of approximately equal mobilities)

(1) *Initial state*: 10mM KCl (12 ml)	*'Cellophane'* $\omega\overline{X} \approx -20$ mM (0·3 mm × 4 cm²)	(2) 1 mM H_4NCl (12 ml)

Time hrs	*Tot.P.D.* mV (2)—(1)	K^+ mM (1)	K^+ mM (2)	H_4N^+ mM (1)	H_4N^+ mM (2)	Cl^- mM (1)	Cl^- mM (2)	$p = e^{\frac{\text{mV}}{25\cdot2}}$	$\frac{(K^+)_1}{(K^+)_2}$	$\frac{(H_4N^+)_1}{(H_4N^+)_2}$	$\frac{(Cl^-)_1}{(Cl^-)_2}$*
0	—	9·88	0	0	1·02	9·90	1·02	—	∞	0	9·8
0^{30}	+47·0	8·94	·66	·60	·47	9·54	1·13	6·46	14·	1·28	8·3
1^{00}	+45·5	8·37	·93	·81	·32	9·18	1·25	6·10	9·0	2·53	7·35
2^{15}	+38·5	7·76	1·34	·92	·25	8·68	1·59	4·61	5·79	3·68	5·46
3^{30}	+32·2	7·72	1·84	·82	·24	8·54	2·08	3·60	4·19	3·42	4·11
4^{50}	+27·7	7·47	2·28	·81	·25	8·28	2·53	3·01	3·28	3·24	3·28
22^{00}	+ 6·1	5·92	4·40	·73	·51	6·65	4·91	1·41	1·34	1·43	1·35

*Note that $Cl^- = K^+ + H_4N^+$

Here two small compartments, both of small volume, were separated by a negatively *charged* membrane, which was *equally* permeable to all ions (in appropriate experiments it was found that the mobilities u_K, u_{H_4N} and v_{Cl} were approximately the same). Accordingly, there exists no reason, in the usual sense, for the development of a DONNAN like distribution phenomenon. Nevertheless the table reveals, that after the system the lapse of some time of rapid ion exchange (particularly of the cations), attains a state characterized by the well known relation $(K^+)_1/(K^+)_2 = (H_4N^+)_1/(H_4N^+)_2$. For the ammonium ions this implies a considerable 'accumulation' on side (1). It is still more important to observe, that these ratios are approximately equal to p, the value demanded by the over-all membrane potential (p is an abbreviation defined $p = \frac{r_2}{r_1} \cdot \xi = e^{(Tot.P.D./25\cdot2)}$). This state is, however, only quasi-stationary, because we see that it slowly declines towards the distribution ratio 1, when both solutions approach identical compositions. An appropriate term for this state would be a 'temporary distribution equilibrium'.

We have here an excellent contribution to the discussion on 'false' and 'real' impermeability (compare p. 308). If we had analysed our model only once, say after about 5 hours, the figures of the ratios $(K^+)_1/(K^+)_2$ and $(H_4N^+)_1/(H_4N^+)_2$ and of the potential function p, would easily have tempted us to give the *false* interpretation that we had met with a genuine DONNAN equilibrium, and hence assumed that the membrane mobility of one of the ions (the Cl^-) was very low indeed or they were completely

impermeable. As the system in this stage changes but slowly with time, a repeated analysis somewhat later would seem to strengthen such views. Such a conclusion is, however, entirely wrong. The whole phenomenon has instead to be referred to the interplay of the *driving forces* and not to restrictions in ability of penetration due to small pore diameters, lack of solubility or similar concepts often applied in biological works. Here the 'trick is played' by the fixed charges or *immobile ions of the membrane matrix*, not by any 'outside' ions! Had the membrane been without charge, so would the equalization of the concentrations have occurred perfectly symmetrically and without any accumulation effects.

The 'pseudo-DONNAN' distributions of the type just described had already been demonstrated by several authors long ago. NETTER[85] did show it in 1928 on dried collodion, and SOLLNER [6,86] has obtained excellent quantitative DONNAN figures on 'permselective' membranes. Both explain, however, their results on the classical impermeability line. STRAUB[87–89] has described similar phenomena on porous systems and has suggested the term 'harmony' for this distribution type. In STRAUB's last publications has he adopted our theory for the 'diffusion effect'. TEORELL[90,91] showed that a living system, namely that of blood-stomach mucosa-gastric juice, could create temporary diffusion effects with regard to the chloride ions, which lead to the suggestion of the 'diffusion hypothesis' for the 'acidity regulation' of the stomach secretion.

A closer analysis of the interdiffusion problem involved in the mammalian stomach secretion is of interest, because it can give a widened insight into the nature of the ionic equilibria under discussion. A model experiment of the blood-gastric acid problem can be arranged with initial conditions as the following (1) 0·1 N HCl (small volume) || membrane, $\omega\bar{X} = 0$ || 0·1 N NaCl (large volume) (2). The result of such an experiment is given in Figure 15. One notices that there takes place a monotonous diffusion exchange between the H^+ and the Na^+ ions. The Cl ions, however, give rise to a transient state (at about 16 minutes), where the chloride net flux is zero (*i.e.* the derivative of the chloride concentration with respect to time, is zero: $d\,\bar{Cl}/dt = 0$). This state really represents a temporary distribution equilibrium, which can be checked up in three different ways. First, the ratio $(Cl^-)_2 : (Cl^-)_1 = 98{:}69 = 1{\cdot}42$ (with correction for activity 1·38), which is almost exactly that demanded by the measured total membrane potential (+ 6·5 mV measured with calomel electrodes in (1) and (2)) according to equation (48) : one gets $\xi = \exp\left(\frac{+6{\cdot}5}{25{\cdot}2}\right) = 1{\cdot}29$. Secondly, the potential simultaneously measured between a pair of AgCl-electrodes (= chloride ion electrodes) in (1) and (2) was approximately *zero* at 16 minutes, as required in a true equilibrium of the Cl ions (see 'Concluding remarks' below). The third

way is a theoretical calculation. At the Cl^- equilibrium one has ϕ Cl = o and the only fluxing ions are those of H and Na with ϕ H = ϕ Na. It can be shown that these conditions lead to a formula for the *temporary ionic distribution equilibrium* (for ωX = o) written

$$\log \xi = \frac{u_H - u_{Ha}}{u_H + u_{Na}} \log \frac{[Na_2]}{[Na_1]} \qquad \ldots . (51)$$

If we employ this equation to the above experiment with $u_H/u_{Na} = 7{\cdot}34$ (same as in free water) and $Na_1 = 59$ ($\gamma_1 = 0{\cdot}80$); $Na_2 = 98$ ($\gamma_2 = 0{\cdot}775$)

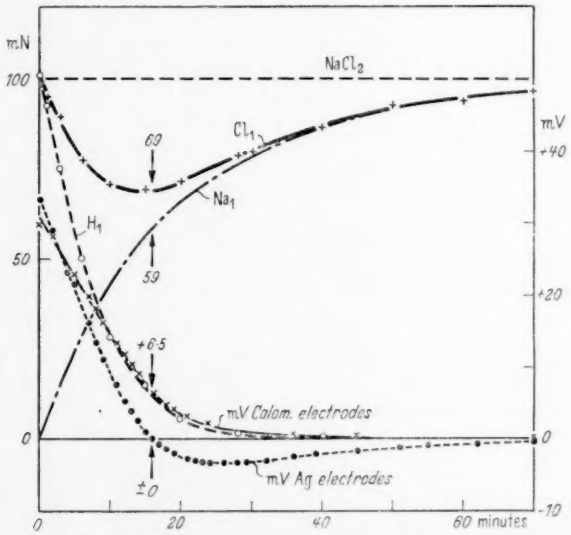

Figure 15. The appearance of a temporary 'diffusion effect' or 'flux equilibrium' in a case of membrane inter-diffusion. (According to an actual experiment by the author)—The initial set up was : (1) o·1 *N* HCl (small volume) || cellophane membrane || o·1 *N* NaCl (large volume) (2). Analyses were performed at different times (crosses and circles) and the membrane potentials were repeatedly measured with a pair of calomel electrodes respectively a pair of AgCl electrodes. Note the temporary zero flux condition (*i.e.* flux equilibrium) for the Cl ions in (1) at 16 minutes.

we obtain $\xi = 1{\cdot}43$, in excellent agreement with the experimental figures given above. This experiment was an example of a temporary ionic depletion and temporary decrease in total concentration *i.e.* in the biological language of osmotic pressure, a *development of a temporary hypotonicity*. A reversed type of experiment with a temporary ionic accumulation has also been described by the author (TEORELL[90], p. 390) and in *Table IV*, the H_4N ions.

Although there was no difference in total concentrations at the initial state, a difference develops in the course of time with a maximum coinciding with the point of Cl^- ion equilibrium (see the Cl curves). This behaviour thus conforming to the 'rule' as regards the necessity of a total concentration difference for the development of 'diffusion effects'.

Electric current flow and ionic distributions—Although most of the foregoing considerations have dealt with cases of free diffusion (electric current flow = 0), it should be emphasized that the basic distribution equilibria equation (48) is perfectly general and valid also when a steady electric current is supplied through the membrane system. In such a case the potential function ξ refers to the potential drop *within* the membrane (at the final steady state). Also under these conditions it is possible for single ion species to attain a state of zero net flux *i.e.* an *equilibrium* state. As an example, to justify this statement, we refer to Figure 10 (p. 335), where the H^+ and Br^- ions show zero net flux at a membrane potential of $+55$ mV, the Na^+ and Cl^- ions at -55 mV (observe that $\log \frac{90}{10} = \frac{55}{58}$ here 90/10 is the ion concentration ratio). In this diagram one should also notice that the *sum* net flux of all cations, or all anions, may be zero at certain potential values. A particular condition occurs when the driving potential is brought to zero; then all ions migrate at rates proportional to their mobilities and the concentration differences. This conforms to a method of 'short-circuiting' employed by USSING[37–39,83] in his interesting studies on the distinction between diffusion forces and 'active transport (compare also LINDERHOLM[64]).

In this connection it is worth of mentioning that HODGKIN and HUXLEY (cited from KEYNES[153]; compare also USSING and ZERAHN[148]) have shown that the partial conductance of the nth ion in *equilibrium* is related to the influx (or the equal outflux) of that ion simply as $G_n = (F^2/RT)(influx)_n$.

Concluding remarks on ionic 'flux equilibria'—It should be emphasized, that the use of the term 'equilibrium' with respect to the ultimate or temporary distribution of the passive ions, attained at zero net flux is justified also from an energetic point of view. As the total membrane potential is equal to the expression for the 'electrode potential' for any passive ion species, a concentration chain consisting of reversible electrodes for such an ion species placed in the solution (1) and (2) should give no current *i.e.* the energy obtained = 0, which we interpret as a sign of a true equilibrium. Thus one has the situation of a system, which as a whole may be a steady state (non-equilibrium), where, however, certain consitutents are in a true state of equilibrium (compare TEORELL[80]). As a proper name for these particular states we suggest the term *flux equilibria*. The term 'diffusion effect' refers to the formation of the characteristic ionic equilibrium distribution by the presence of a diffusion flow, just as

the term 'DONNAN effect' refers to the formation of an analogous ion distribution owing to the presence of impermeable ionic constituents. An electric current flow does also involve possibilities for individual ionic equilibria, as pointed out in the previous section.

Flux equilibria are perhaps not common in *in vitro* chemistry, but they may presumably be of importance in biological systems. Their special feature is the production of unequal cation and anion distributions, but it must be stressed, that they do not allow for ionic selectivity like the potassium-sodium accumulation-depletion so commonly met with in living structures. Another limitation is, that they may require differences in total osmotic pressure between the 'inside' and 'outside' solutions, which, as a rule, are rather small within the living bodies. Further extensions of the theory for flux equilibria are, however, possible. Promising aspects might arise from considerations, for instance, of the modifying effects of presence of ions with higher valencies, of simultaneous flow of water, of chemical reactions superposed on the diffusing systems and of coupling to electron transferring mechanisms.

6. *The spatial distribution of ions and potential within membranes*

In a previous section it has been emphasized, that the development of any membrane permeability theory must in fact rest on some assumption or other as to the mode in which the ions and/or the potential are distributed in a diffusion layer (compare pp. 317, 327). The simple HENDERSON assumption of 'linear mixtures' stands, for instance, against the more intractable PLANCK concepts of a 'constrained layer'. An excellent discussion on these matters can be found in MACINNES' book[92] (p. 231). There is, however, little doubt that the PLANCK view is the physically true one in *all* cases, a conviction that caused us to revise and extend the original, provisional 'fixed charge theory' (TEORELL[19], compare p. 318). The extended theory has also the feature, that it allows an explicit calculation of the course of all ion concentrations within the membrane, as well as of the course of the electric potential. A diagram showing some experimental and theoretical results is shown in Figure 16. We shall here refrain from a presentation of the underlying equations, since they can be found in the paper quoted, ([19]p. 468), and instead point out a few interesting features :

The *total* concentration does not need to vary in a straight line manner in charged membranes as it is known to do in uncharged diffusion layers (compare Figure 6, the $C\bar{l}$-curve). The *individual* ion concentrations do generally show a curved course. Under certain circumstances even *concentration maxima may occur inside the membrane*, and the average concentration within the membrane of the ion in question may be higher than in the surrounding bulk, thus simulating a specific 'adsorption' to the membrane! (compare Figure 16, the Na-curve). A few other, peculiar

membrane distributions of protein ions have been mentioned by TEORELL[145] (p. 1141-42) and are reproduced in Figure 17.

The *potential* in the *interior* of the membrane has in general an exponential-like course. Compare the mV-curve of Figure 16. An exception

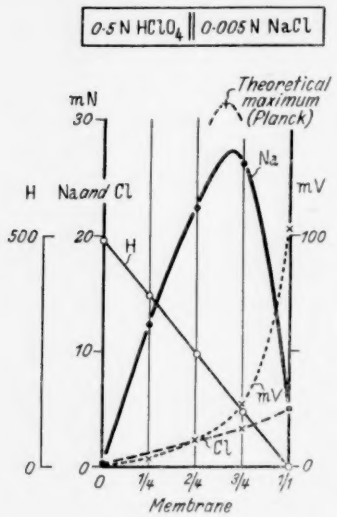

Figure 16. The spatial distribution of the ions and the potential within a membrane in a case of interdiffusion (uncharged cellophane). The points were determined analytically with the aid of a 'multimembrane' arrangement (according to TEORELL, *J. biol. Chem.* 113 (1936) 735). The results conform reasonably well to the theoretically calculated curves (according to PLANCK-PLETTIG).

is the case where the surrounding *total* concentrations are equal, when a 'constant field' as well as a constant total concentration may be present throughout the membrane (compare p. 331, and TEORELL[35], p. 211). The DONNAN potential components appear as jumps at the phase boundaries; an example can be found in Figure 6 (p. 326).

8. DISCUSSION ON THE VALIDITY OF THE FIXED CHARGE THEORY AND ON SOME MODIFYING FACTORS

The fixed charge theory has hitherto been tested experimentally only in some limited parts, in the main by measurements of electrical potential and by a few conductance experiments. Experimental data suitable for comparisons with the extended theory outlined above, which also takes care of the ion fluxes, distribution equilibria *etc.* are very few as yet. It is therefore somewhat premature to state anything definite about the practical applicability of the new quantitative expressions, which have been derived. Certainly, the confrontation of the theory with actual experiments and findings will reveal that the fundamental assumptions used are, in many cases, too simplified. It is hoped, however, that the theory in its present state may be suitable for further amplification and that it can serve, at least, as a working hypothesis for the experimentalists.

Some of the more obvious weaknesses and deficiencies of the theory can, however, be discussed somewhat in advance, while one awaits the carrying out of necessary experiments. A great deal of useful information

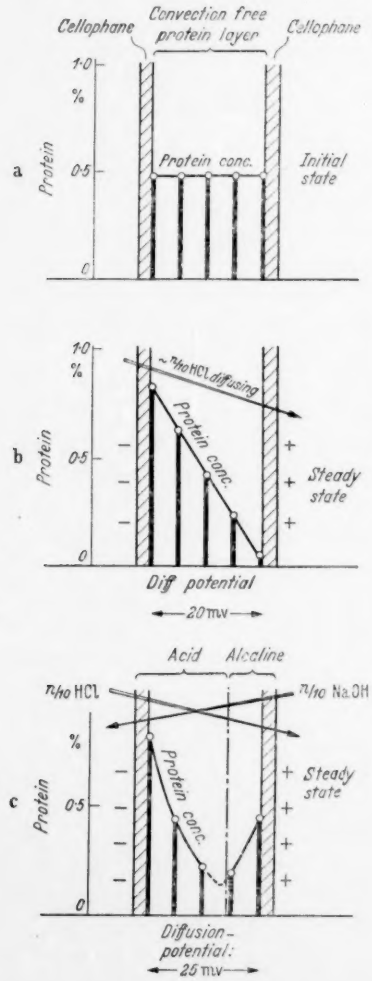

Figure 17. The spatial distribution of ionized protein within a porous, uncharged membrane. The membrane *surfaces* are here impermeable to the protein. A steady diffusion stream of a 'diffusing agent', HCl or NaOH, gives rise to a potential across the membrane system, which causes a marked redistribution of the protein ions (the black bars), compare Figure 13.—(*a*) The initial state with a constant protein distribution. (*b*) The final steady state attained with HCl as a diffusing agent. The protein ions are here positive. (*c*) The steady state attained with two diffusing agents, HCl and NaOH, diffusing across the membrane in opposite directions. The peculiar protein distribution is caused by the fact that the protein particles are positive to the left and negative to the right, because of differences in *p*H (after unpublished experiments by the author).

can be obtained from the rapidly growing literature on ion exchange material, which naturally has to treat kindred problems.

1. *The structure and chemical nature of the membrane*

The fixed charge theory in its present form assumes that the membrane matrix contains water spaces and is homogeneous throughout and that

the spatial distribution of the fixed, ionizing groups (or adsorbed groups or polar groups), is constant in every part. This volume density of the charge has been arbitrarily reduced to an average but constant 'membrane ion concentration' ($\omega\overline{X}$) (synonyms are 'Festionen-Konzentration' or 'selectivity constant'). Nothing more has to be assumed as to the structure. The present knowledge of the actual architecture of artificial membranes conforms to these assumptions. The electron-microscope has revealed a net or web configuration with interspaced pores of width from 0·15 — 1 μ (HAUSMANN and PIETSCH[93]). Various other techniques also indicate that the pore size may be rather homogeneous (compare MANEGOLD[94]). WIRTH[95] claims that cellophane has a mean pore width of only 20 $\mu\mu$ *i.e.* allowing space for about 200 water molecules, the free pore area is found to occupy only 6 per cent of the total diaphragm surface. No absolute figures seem to be accessible as yet for the modern ion exchange resins, but KUNIN[96] reports that the capacities of various cation exchange resins decrease as the ionic size of the cations attains a threshold value. On the other hand, it can be estimated that the thickness of the diffuse double layer outside the charged points is of the order 100-1000 Å. This shows that there may exist an overlapping of the double layers inside the membrane. At any rate, it seems that the fixed charge distribution may be submicroscopical enough to allow the use of a symbolic 'membrane ion concentration' expressed in the usual units, as gram ions per litre.

Should it happen that very wide pores exist side by side with very narrow ones, some disturbing effects are to be expected, because the central 'threads' of the solution in such coarse pores will escape from the wall effects and become 'leaky'. This effect has been emphasized by SOLLNER[97], who also stresses that the 'fixed charge' control of the ion migration may be confined to a few critical points in heteroporous membranes. He suggests, that the assumption of heteroporosity affords, at least qualitatively, a satisfactory explanation of some observed discrepancies from the fixed charge theory.

The ionization strength of the 'membrane electrolyte' is of course of great importance. In the fundamental assumptions of the present fixed charge theory (p. 318) the postulate of a strongly acidic or basic character of the membrane matrix is included. This was necessary in order to secure a constant ionization of the membrane electrolyte, independent of the degree of dilution of the surrounding salt solutions *i.e.* to prevent 'membrane hydrolysis'. But if one meets with weaker membrane electrolytes, or with amphoteric membranes, one has to expect effects from 'membrane hydrolysis' and pronounced effects of *p*H *etc.* The direction of the influence of dilution of the surrounding media will be a decrease of the effective membrane charge. Here the present theories require further extensions.

The influence of asymmetrical membranes is very difficult to overlook. CRANK[99] has treated the diffusion in media with variable properties. His results are, however, not applicable to ionic transport in charged membranes. Our present assumption of a constant ionisation or charge throughout the membrane is certainly not valid in many membranes. Even simple extensions as the postulation of a 'skew' but linear course of the membrane charge meet with difficulties not yet solved. A relevant problem is the diffusion with 'non-linear adsorption' outlined by CRANK[98]. MARSHALL[100], working with mineral membranes has recently modified the TEORELL-MEYER-SIEVERS concepts to account for the case of absence of water inside the membrane structure.

A great number of other interesting combinations of the membrane properties can be thought of. The classical "mosaic membrane" of a composite of positive and negative parts in juxtaposition is of great biological interest and has recently been considered at hand of some model experiments by NEIHOF and SOLLNER[101]. In these combinations one may achieve local currents of electricity and many odd ionic distribution effects. Membranes of 'sandwich' type may also exist. It is obvious that these more involved membrane systems offer many important problems which challenge the theoreticians as well as the experimentalists.

2. *The thickness effect*—The crucial point in the fixed charge theory is the assumption of a fully developed DONNAN mechanism at the membrane interfaces, which tacitly implies, that the rate of transport is entirely controlled by the migration rate in the interior of the membrane. This view is certainly a good approximation at *thick* membranes and with well stirred outside solutions. The important questions may now be raised: under what circumstances do these assumptions break down; does there exist a lower critical thickness, where the 'boundary process' takes over the rate control? One would expect some limit, arguing that with decreasing thickness would the 'membrane process' be increasingly faster and finally overtaking the boundary processes of ion exchanges.

A discussion of the thickness effect may be carried out along three lines (*a*) the nature of the boundary process, (*b*) the kinetics of the boundary process, (*c*) the rate of the membrane process.

(*a*) *The nature of the boundary process* has been regarded hitherto as an establishment of some form of adsorption equilibrium or a DONNAN equilibrium. Although the type of equilibrium existing does not necessarily tell us anything about the rate processes, it is of a vital interest to mention briefly this subject. Workers on the natural ion exchangers (zeolite and other minerals, clays *etc.*) have suggested either an 'exchange adsorption' mechanism (in particular JENNY[102], JENNY and OVERSTREET[103]) or a DONNAN distribution (MATTSON and his school[104], see for instance WIKLANDER[105]). The DONNAN mechanism seems to have gained in popularity in recent

years, probably due to its usefulness in the synthetic ion exchangers (WIKLANDER[106]). A survey of the exchange processes at this group of substances, which probably can be regarded as prototypes also for many living membranes, is presented in the recent monographs of NACHOD[11] and KUNIN and MYERS[12]. On the whole, it appears that a DONNAN equilibrium is generally established in the simplest cases of exchange with single alkali salts. In mixtures of ions there may sometimes appear a more or less pronounced selective affinity (due to ionic radius, ionic hydration *etc.*; *cf.* the section 'ion selectivity' below).

(*b*) Now turning again to the *kinetics of the boundary ion exchange*, it is first obvious that a very efficient convection (stirring) in the surrounding solutions is important, a fact which has sometimes been overlooked. SCHULMAN and TEORELL[107] and MANEGOLD and KALAUCH[108] report 'unstirred layers' of the order 10-30μ which can affect the results. As regards the boundary process proper, there are somewhat shifting opinions. HALE and REICHENBERG[109] advance the view, that the outside ion concentrations are of importance for the exchange kinetics ($Na^+ \rightleftarrows H^+$ —*resin*): at *low* concentrations of Na^+ ions in the solution, the rate-determining mechanism is the diffusion of ions through the thin film of liquid surrounding the resin particle, at *high* Na^+ concentrations the rate control may reside inside the particle. Similar discussion are given by ADAMSON and GROSSMAN[110]; BOYD, ADAMSON and MYERS[111] and in great detail by KRESSMAN and KITCHENER[112] and others. The ion exchange mechanisms have also recently been treated with statistical-mechanical equations by KRISHNAMOORTHY and OVERSTREET[113], and according to the oscillation volume theory of JENNY by DAVIS[114]; experiments on the exchange of isotopic ions have been performed among others by HOLM and WESTERMARK[115]. CRISP[116] makes the sensible suggestion, that exchange studies could profitably be carried out on surface film systems. J. T. DAVIES[118] has recently attempted such a study on insoluble charged surface films. DAVIES[117] also made a thorough theoretical and experimental study of diffusion at liquid-liquid phase boundaries and claims, that a phase boundary permeation may be 10^6 times slower than in free water. This fits in with the 'transient state theory' of DANIELLI and of EYRING *et al.* cited above (p. 313). There may exist high 'free energy barriers' at the interface, particularly when there is a different solvent in the membrane, which slows down the rate of passage of the ions.

In this connection an analysis according to the DANIELLI-EYRING concepts is very instructive (compare p. 313): The *general* permeability constant is in DANIELLI's notations (*cf.* Eq. (3)) equal to $P = ae/(nb+2e)$. For *thick* membranes is $nb \gg 2e$ and $P \approx (a/b)\cdot(e/n)$, hence the over-all penetration rate is controlled by the membrane process factor e, the partition coefficient a/b and the membrane thickness factor n. This

implies that the rate of the interfacial process is the more rapid one (allowing adjustments to equilibrium states at the membrane interface *i.e.* an establishment of a full DONNAN distribution). For *thin* membranes $nb \ll 2e$ and $P \approx a/2$, which means that the over-all rate will be controlled entirely by the a factor belonging to the 'boundary process'. DANIELLI has also claimed, that the fixed charge theory, in the version of TEORELL-MEYER-SIEVERS, can only be applicable to the thick membranes[2] (p. 330). Where the limit of thickness comes, still remains to be found out.

The important conclusion, which can be extracted from our discussion of some relevant papers, is that *there can and must exist conditions, where the boundary process can be slowest* and hence interfacial equilibria conditions occur different from those used as basic concepts in the fixed charge theory. *Discrepancies with the theory* have therefore to be expected at very *thin* membranes, and probably also *when the concentration jumps at the interfaces are very large i.e.* in cases where the membrane fixed ion concentration is much *higher* than the surrounding ion concentrations ($\omega\overline{X} \gg a$).

In this connection, it deserves to be pointed out that the *partition coefficients* employed at liquid-liquid interfaces denote nothing but the ratio of 'forward rate' to 'backward rate' of the solute at *equilibrium*. It is obvious that the equilibria constants ξ, p and r_1 and r_2 employed in the electrolyte membrane system of this paper have a *formal* correspondence to partition constants. Hence, it is natural that the equations describing the diffusion kinetics should come out in similar forms regardless of the nature of the membrane, whether a 'solubility' or 'oil' membrane is present or one with a solid matrix supporting a 'connecting link' of aqueous solution. Our formula (7) for the flux $\phi^{+\prime} = -K^{+}u'\,(c_2{}^{+\prime}\xi - c_1{}^{+\prime})$ can be written as $\phi = +k_1c_1 - k_2c_2$, which, in fact is identical with, for instance, DAVIES (*loc. cit.*) equation (1), for the flux across a H_2O-oil interface, where k_2/k_1 is the partition coefficient (corresponding to the ξ).

(*c*) The '*membrane process*' as studied in artificial resin particles appears actually to be quite slow in many instances. GREGOR, COLLINS and POPE[119] claim that neutral molecules in a sulfonic acid resin have a diffusion coefficient of the order 0·002 – 0·1 of that in free water (compare p. 337). Some important aspects, when discussing the slow migration processes within a charged membrane, are no doubt the *activity* conditions of mobile and fixed ions and the *selectivity* properties. In the modern exchange resins it is possible to obtain a membrane concentration $\omega\overline{X}$ of strength up to about two molar. Even with much smaller concentrations, it is highly probable that inter-ionic attraction effects will be noticeable. Steric factors *etc.* will also be of importance in these respects. We shall not go into more discussion, but instead limit ourselves to referring to

some recent publications which are worth consulting:—An application of the interionic attraction theory to the diffuse layer around colloid particles is given by LOEB[120]. Theories and experiments aiming to the determination of thermodynamic quantities as activity constants, *etc.* in exchange resin can be found in articles by BOYD, ADAMSON and MYERS[111]; DUNCAN and LISTER[121]; COSGROVE and STRICKLAND[122]; ARGERSINGER, DAVIDSON and BONNER[123]; HÖGFELDT, EKEDAHL and SILLÉN[124]; BABCOCK, DAVIS and OVERSTREET[160]. From these papers the fact emerges that *it may be necessary to introduce appreciable corrections for activity* in the fixed charge theory.

3. *The ion selectivity and the fixed charge theory*

As regards ion *selectivity* it can be said that the experience with ion exchange resins shows that it can be more or less marked depending on the experimental conditions; in particular it appears that the chemical structure is important (the strength of the membrane 'acid' or 'base', the degree of cross-linking *etc.*). GREGOR, BREGMAN *et al.*[125] in a careful investigation on the resin 'Dowex 50' report a rather uniform exchange *capacity* for eight common ions, the exchange *rate* can, however, be quite different. This selectivity is, according to GREGOR and BREGMAN[126], a function of the activity in the resin phase, of the ionic volumes *etc.* These authors especially emphasize, that the resin matrix may exhibit *swelling* (which is disregarded in the fixed charge theory). MORTON[127] working with 'Zeo-Karb 225' finds a high affinity for a bivalent ion (Ca), less for K and still less for Na. SOLLNER[128,129] and SOLLNER and GREGOR[130,131] discuss in some detail the ion selectivity problems in the permselective membranes and it is appropriate to state that SOLLNER's views are of great interest for the fixed charge theory. WIKLANDER[106] has treated the influence of varying proportions of the exchanging ions. Further papers dealing with the selectivity of ion exchanges are, for example, those by DEUEL *et al.*[132]; AUSTERWEIL[133]; see also the monographs of NACHOD[11] and of KUNIN and MYERS[12]. A stimulating general article on the limiting factors of permeabilities in membranes has been published by KUHN[134]. The problem of *weak* electrolyte mixtures (not included in the fixed charge theory) has been treated by C. W. DAVIES[135].

In *conclusion* it can be said that it seems as if it is not permissible to assume tacitly the *same* distribution ratio at the membrane interfaces for all univalent ions as is done in the extended fixed charge theory. In other words, one has to apply with some caution the DONNAN concept which assumed that membrane surface concentrations c and bulk concentrations a are related as

$$r = c^{+\prime}/a^{+\prime} = c^{+\prime\prime}/a^{+\prime\prime} \ldots = c^{+}/a^{+} = a^{-}/c^{-} = a^{-\prime}/c^{-\prime} = a^{-\prime\prime}/c^{-\prime\prime} \quad (8)$$

This restriction may at the first sight appear as a serious objection menacing the whole theory. However, this is not the case, because the core of *the extended theory operates with entirely arbitrary values of the interfacial mobile ion concentrations* but with the electroneutrality condition that $(c^- - c^+ - \omega\bar{X}) = 0$. These quantities, $c_1{}^{+\prime}$, $c_2{}^{+\prime}$ . . . $c_1{}^{-\prime}$, $c_2{}^{-\prime}$ . . . *etc.* entering in the basic NERNST-PLANCK equation (1:1,4) can be retained in *all* the resulting formulas, although they have in some cases above been substituted by the equivalent forms $a^+ \cdot r$, or a^-/r. The DONNAN relations were chosen as being the simplest and they seem to correspond well to the actual physical event in a great many cases. *There is, however, nothing to prevent the choice of any other theoretical or empirical relation between the bulk and membrane interface concentration of the mobile ions.* Practically all the formulas derived can still be explicitly solved if one obtains some means of substituting the DONNAN ratios r_1 and r_2 *by individual ion distribution ratios* (as $\rho^{+\prime} = c^{+\prime}/a^{+\prime}$, $\rho^{+\prime\prime} = c^{+\prime\prime}/a^{+\prime\prime}$ . . . $\rho^{-\prime} = a^{-\prime}/c^{-\prime}$ *etc.*). In fact, this possibility was utilized by MEYER, HAUPTMANN and SIEVERS[136] in their version of the fixed charge theory to include water-oil membrane potentials. The calculation of the *total* membrane potential, particularly in electrolyte mixtures, may, however, present difficulties. The definition of the components of the phase-boundary potentials (corresponding to the DONNAN potentials) needs a careful consideration, but the intermembrane component can be defined as before.

A further examination of the consequences of ion exchange selectivity would be of the greatest biological interest, it might offer some connecting links to the understanding of the selective accumulation of for instance K over Na, common in many living cells. Much help is to be expected from the field of ion exchangers; thus KUNIN and MYERS[12] (p. 63), report that SKOGSEID has succeeded in synthesizing a resin with an unusual affinity for the potassium ion.

4. *The influence of water flow*

Freedom from convections was assumed in our presentation of the fixed charge theory. In many types of artificial membranes the experimental findings justify this postulate, but in the living membranes it may be necessary to account for the effects of a bulk flow of the solution within the membranes. This bulk or mass flow can arise from differences of osmotic pressure or/and of hydrostatic pressure, or from electro-endosmosis. A general treatment of diffusion processes with superposed mass flow needs a careful definition of a 'frame of reference' as emphasized by HARTLEY and CRANK[137], who also derive an expression for the mutual diffusion coefficients in a binary solution, when the effect of an over-all mass-flow of the solution is included.

It is comparatively easy to get some ideas of the influence of a water flow superposed on an electrolyte diffusion system. One has to supplement the basic flux expression with an additional term $\mu \cdot c^{+\prime}$, where μ is the velocity of the water flow (compare WILLIAMS and CADY[138], p. 181, BLOCK[139], RASHEVSKY[140], p. 13, TEORELL[19], *Gl.* (15); also USSING[39] who employed the driving force of the water transport instead of resulting velocity). One gets from our equation 1·3, for a *cation flux*

$$\phi^{+\prime} = -RT \cdot u' \cdot c^{+\prime} \cdot \frac{d\,(\ln c^{+\prime} \cdot \xi)}{dx} + \mu\, c^{+\prime} =$$

$$= -RT \cdot u' \cdot c^{+\prime} \left[\frac{d\,(\ln c^{+\prime} \cdot \xi)}{dx} - \frac{d\,(\mu x / RTu')}{dx} \right] \quad \ldots . \quad (52{:}1)$$

and with introduction of the 'water' potential function, in analogy with ξ, defined as $\eta' = \exp\,[(-\mu x/RTu')]$ one obtains $(-\mu x/RTu') = ln\ \eta'$, which introduced in (52:1) yields

$$\phi^{+\prime} = -RT \cdot u' \cdot c^{+\prime} \cdot \frac{d\,[\ln (c^{+\prime} \cdot \xi \cdot \eta')]}{dx} \quad \ldots . \quad (52{:}2)$$

The integration of this equation, for a charged membrane, remains to be done for the general case. Some important information as to the ionic distribution equilibria can, however, be obtained by a study of the relative flux relations (compare p. 345). In order to form the in-out flux ratio of a single cation species one can regard the inward flux as provisionally belonging to one species, ($\phi^{+\prime}$) and the outward flux as belonging to another species ($\phi^{+\prime\prime}$) of the *same* mobility ($u' = u'' = u$), for instance the isotope of the first! By dividing two equations (52:2) one can derive (with $\eta' = \eta''$ and $c_1{}^{+\prime} = 0$; $c_2{}^{+\prime\prime} = 0$ and restoring $c_1{}^{+\prime\prime}$ with the identical $c_1{}^{+\prime}$)

$$\frac{influx}{outflux} = \frac{\phi^{+\prime}}{\phi^{+\prime\prime}} = -\frac{c_2{}^{+\prime} \cdot \xi \cdot \eta_2'}{c_1{}^{+\prime}} = -\left(\frac{a_2{}^{+\prime}}{a_1{}^{+\prime}}\right) \cdot \left(\frac{r_2}{r_1} \cdot \xi \cdot \eta_2'\right) \quad \ldots . \quad (53)$$

Equation (53) is obviously an extension of *in-outflux equation* (41). It is interesting to note that all factors r_1, r_2, ξ and η refer to various types of potentials. USSING'S version of the in-outflux ratio, for an uncharged membrane, is essentially similar to (53). In the state of a *flux equilibrium* is the in-outflux ratio equal to 1 and (53) for a cation species, is transformed to

$$\left[\frac{c_1{}^{+\prime}}{c_2{}^{+\prime}}\right]_{\text{equil.}} = \xi \cdot \eta_2' \quad \text{or} \quad \left[\frac{a_1{}^{+\prime}}{a_2{}^{+\prime}}\right]_{\text{equil.}} = \left(\frac{r_2}{r_1} \cdot \xi\right) \cdot e^{\frac{-\mu\delta}{RTu'}} \quad \ldots . \quad (54{:}\ 1,\ 2)$$

If one compares this equilibrium with the one described by expressions (48) it is seen that the equilibrium distribution ratio, as determined by the

resulting electrical potential will be changed by a factor of η'_2 equal to $e^{-\mu\delta/RTu'}$. The system will always *strive* to approach this ratio. In confined volumes of the outside electrolytes this theoretical ratio, however, will never be actually attained, unless the water content somehow remains constant. The theoretical equilibria are nevertheless of interest because the departure of the system from its equilibrium state influences the kinetics of the ionic transport. Remembering that $\xi = e^{F(\varphi_2 - \varphi_1)/RT}$ and $\frac{r_2 \cdot \xi}{r_1} = e^{F(Tot.P.D.)/RT}$ one realizes that *the effect of the water flow is equivalent to the superposition upon the electrical potential of another extra potential.* It is perhaps more elucidating to describe the balance at equilibrium by generalizing equation (54) in the logarithmic form

$$RT \ln \left[\frac{a_1^{+\prime}}{a_2^{+\prime}}\right]_{\text{equil.}} = F \cdot (Tot.P.D.) - \frac{\mu\delta}{u'} + (\textit{extra work terms}) \quad \ldots . \quad (55)$$

Here $RT \cdot \ln(a_1^{+\prime}/a_2^{+\prime})$ is known as the 'osmotic work', the term $F \cdot (Tot.\ P.D.)$ is the 'electrical work' and the water flow term $\mu\delta/u'$ has the dimensions of a 'mechanical work'. *Any other force which might appear in the system will obviously add as a new work term in* (55), *or as an extra factor expressing the corresponding potential function in* (53) *or* (54). One has, for instance, the chemical work implied in 'active transport' (compare the papers by USSING and ZERAHN[143] and LINDERHOLM[64]). Also other energy forms are conceivable in the biological systems such as surface energy *etc.* In this connexion it may be referred to the interesting treatments of the energetics of steady states by REINER and SPIEGELMAN[141] and by ROSENBERG[142].

It may be emphasized that the existence of a water flow may induce individually different accumulation effects upon the ions present, because the water flow effect is dependent on a common velocity μ, and on *individual* mobilities u', u'' *etc.* This is an effect related to the HERTZ principle (HERTZ[143], JACOBS[144], p. 134, TEORELL[146], p. 1019; [19], p. 464). Such different ion selective accumulation effects do not follow from any kind of the simpler 'diffusion effects' discussed earlier.

5. *Electro-endosmosis, streaming potentials, etc.*

When *mobile* ionized charged particles are placed in an electrical field one gets a migration of those relative to the solvent *i.e.* the well known process of *cataphoresis* or *electrophoresis.* Now if the particles are collected together and 'sintered' into a porous, fixed membrane one should obtain the same *relative* migration *i.e.* the water has to move across the membrane, as in the phenomenon of *electro-endosmosis.* If the solution by some means is moved through the membrane there appears instead an electric potential,

i.e. the *streaming potential.* According to the classical concepts of HELMHOLTZ and others it is the 'double layer' close to the pore walls which causes the endosmosis to occur. The electrokinetical effects are usually expressed in terms of a wall charge and given as the zeta-potential, ζ. Previously it has been pointed out, that the common double layer concepts break down in membranes with *very fine pores*, where an 'overlapping' of the double layer must take place. Such ideas have been mentioned, for instance by LANGE and NAGEL[147], and in considerable detail recently by SCHMID *et al.*[59–62]. SCHMID imagines, that the 'wall charges' in such submicroscopic pores can be considered as an approximately homogeneous electrolyte with immobile ions. But this is exactly the essence of the TEORELL-MEYER-SIEVERS 'fixed charge theory', (compare 'The effect of the membrane is regarded as that of an added electrolyte', TEORELL[25]). SCHMID[59,60,154] develops on this basis a number of new formulas. In SCHMID's expressions the '*Festionen-Konzentration*' have replaced the zeta potentials and he can satisfactorily account for his own and other investigations as regards electro-osmosis, membrane conductance (compare p. 336) streaming and dialysis potentials. SCHMID's 'Festionen-Konzentration' is identical with MEYER-SIEVERS 'selectivity constant A' and TEORELL's $\bar{X}$ or $\omega\bar{X}$, and has the dimensions of a concentration. It is of course directly related to the so called 'exchange' capacity' used in the field of ion exchangers.

It is quite obvious, that SCHMID's attempt to extend the fixed charge theory to include the electrically induced water flow, is of the utmost importance also for those dealing with biological systems, where the water transport is as striking a phenomenon as the molecular, or ionic transport. The opening of the field by SCHMID thus appears very promising.

Other recent papers worth mentioning in this connection are the following : CAUSSE[149] has reviewed electro-kinetic phenomena; MAZUR and OVERBEEK[150] have treated the quantitative relation between electrokinetic effects. The related procedure of electro-dialysis is naturally influenced by the charge of the dialysis membranes employed, particularly with regard to *p*H changes. Such aspects have been again considered by KALAUSH[151], compare also a paper on simultaneous dialysis of ions by HACKER and RECHMAN[152].

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